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(54) Title: NOVEL P-SELECTIN LIGAND PROTEIN (57) Abstract A novel P-selectin ligand glycoprotein is disclosed, comprising the amino acid sequence set forth in SEQ ID NO:2 or by the amino acid sequence set forth in SEQ ID NO:4. DNA sequences encoding the P-selectin ligand protein are also disclosed, along with vectors, host cells, and methods of making the P-selectin ligand protein. Pharmaceutical compositions containing the P-selectin ligand protein and methods of treating inflammatory disease states characterized by P-selectin- and E-selectin-mediated intercellular adhesion are also disclosed.		

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NOVEL P-SELECTIN LIGAND PROTEIN

This application is a continuation-in-part of copending applications U.S. Serial No. 08/316,305, filed September 30, 1994, which was a continuation-in-part of copending application U.S. Serial No. 08/235,398, filed April 28, 1994, which was a continuation-in-part of copending application U.S. Serial No. 08/112,608, filed August 26, 1993, which was a continuation-in-part of U.S. Serial No. 07/965,662, filed October 23, 1992, now abandoned. This application also claims priority from International Application No. PCT/US93/10168, filed October 22, 1993.

BACKGROUND OF THE INVENTION

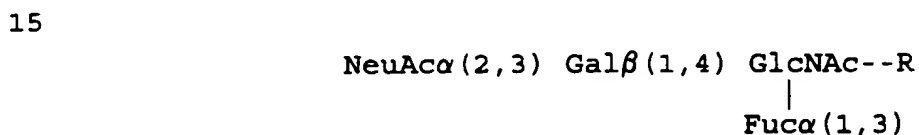
The present invention relates to the field of anti-inflammatory substances which act by inhibiting leukocyte adhesion to endothelial cells. More particularly, the present invention is directed to novel ligands for the mammalian adhesion proteins known as selectins.

During inflammation leukocytes adhere to the vascular endothelium and enter subendothelial tissue, an interaction which is mediated by specific binding of the selectin or LEC-CAM class of proteins to ligands on target cells. Such selectin-mediated cellular adhesion also occurs in thrombotic disorders and parasitic diseases and may be implicated in metastatic spread of tumor cells.

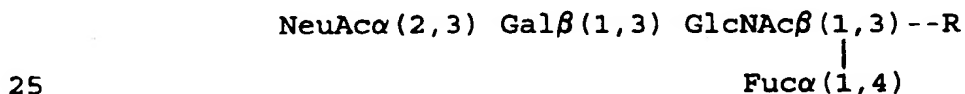
The selectin proteins are characterized by a N-terminal lectin-like domain, an epidermal growth factor-like domain, and regions of homology to complement binding proteins. Thus far three human selectin proteins have been identified, E-selectin (formerly ELAM-1), L-selectin (formerly LAM-1) and P-selectin (formerly PADGEM or GMP-140). E-selectin is induced on endothelial cells several hours after activation by cytokines, mediating the calcium-dependent interaction between neutrophils and the

endothelium. L-selectin is the lymphocyte homing receptor, and P-selectin rapidly appears on the cell surface of platelets when they are activated, mediating calcium-dependent adhesion of neutrophils or monocytes to
 5 platelets. P-selectin is also found in the Weibel-Palade bodies of endothelial cells; upon its release from these vesicles P-selectin mediates early binding of neutrophils to histamine-or thrombin-stimulated endothelium.

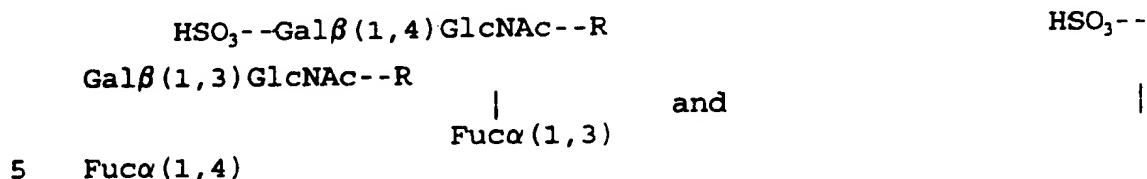
Selectins are believed to mediate adhesion through
 10 specific interactions with ligands present on the surface of target cells. Generally the ligands of selectins are comprised at least in part of a carbohydrate moiety. For example, E-selectin binds to carbohydrates having the terminal structure



20 and also to carbohydrates having the terminal structure



where R =the remainder of the carbohydrate chain. These
 30 carbohydrates are known blood group antigens and are commonly referred to as sialyl Lewis^x and sialyl Lewis^a, respectively. The presence of the sialyl Lewis^x antigen alone on the surface of an endothelial cell may be sufficient to promote binding to an E-selectin expressing
 35 cell. E-selectin also binds to carbohydrates having the terminal structures



10 As with E-selectin, each selectin appears to bind to a range of carbohydrates with varying affinities. The strength of the selectin mediated adhesive event (binding affinity) may also depend on the density of the carbohydrate and on the density of the selectin on the cell surface.

15 P-selectin binds to carbohydrates containing the non-sialated form of the Lewis^x blood group antigen and with higher affinity to sialyl Lewis^x. P-selectin may also recognize sulfatides, which are heterogeneous 3-sulfated galactosyl ceramides, isolated from myeloid and tumor cells by lipid extraction. However, the binding of cells bearing P-selectin to cells bearing P-selectin ligands is abolished when the ligand-bearing cells are treated with proteases, indicating that the P-selectin ligand may be a glycoprotein.

20 Two putative glycoprotein ligands for P-selectin have recently been identified, one of which has been partially purified, (Moore et al., J. Cell Biol. 118, 445-456 (1992)). However, neither amino acid composition nor the amino acid sequence of these glycoproteins are disclosed.

SUMMARY OF THE INVENTION

25 In one embodiment, the present invention provides a composition comprising an isolated DNA encoding a P-selectin ligand protein, said protein comprising the amino acid sequence set forth in SEQ ID NO:2 from amino acid 1 to amino acid 402. Also provided is a composition comprising an isolated DNA encoding a soluble P-selectin ligand protein, said protein comprising the amino acid sequence

set forth in SEQ ID NO:2 from amino acid 1 to amino acid 310. The invention further provides a composition comprising an isolated DNA encoding a mature P-selectin ligand protein, said protein comprising the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 402. In another embodiment, the invention provides a composition comprising an isolated DNA encoding a soluble mature P-selectin ligand protein, said protein comprising the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 310. In another embodiment, the invention provides a composition comprising an isolated DNA encoding a P-selectin ligand protein, said protein comprising the amino acid sequence set forth in SEQ ID NO:4. The invention further provides a composition comprising an expression vector comprising any one of the isolated DNAs of the invention, said DNA being operably linked to an expression control sequence; a host cell transformed with the expression vector containing any one of the DNAs described above; and a process for producing the P-selectin ligand protein, which comprises:

- (a) culturing a host cell transformed with an expression vector containing any one of the DNAs of the invention in a suitable culture medium; and
- (b) purifying the P-selectin ligand protein from the culture medium.

In another embodiment, the invention provides a composition comprising a protein comprising the amino acid sequence set forth in SEQ ID NO:2 from amino acid 21 to amino acid 402, said protein being substantially free from other mammalian proteins. The invention further comprises a soluble P-selectin ligand protein comprising the amino acid sequence set forth in SEQ ID NO:2 from amino acid 21 to amino acid 310, said protein being substantially free from other mammalian proteins. In another embodiment, the invention comprises a P-selectin ligand protein comprising the amino acid sequence set forth in SEQ ID NO:2 from amino acid 1 to amino acid 402, said protein being substantially

free from other mammalian proteins. The invention also provides a composition comprising a mature P-selectin ligand protein comprising the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 402, said protein being substantially free from other mammalian proteins. Further provided is a composition comprising a soluble mature P-selectin ligand protein comprising the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 310, said protein being substantially free from other mammalian proteins. In another embodiment, the invention provides a composition comprising a protein comprising the amino acid sequence set forth in SEQ ID NO:4.

In yet another embodiment, the invention provides compositions comprising antibodies specific for P-selectin ligand proteins.

In another embodiment, the invention provides a method of identifying an inhibitor of P-selectin-mediated intercellular adhesion which comprises:

(a) combining a P-selectin protein with a P-selectin ligand protein comprising an amino acid sequence selected from the group consisting of the amino acid sequence set forth in SEQ ID NO:2 from amino acid 1 to amino acid 402, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 402, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 310, and the amino acid sequence set forth in SEQ ID NO:4, said combination forming a first binding mixture;

(b) measuring the amount of binding between the P-selectin protein and the P-selectin ligand protein in the first binding mixture;

(c) combining a compound with the P-selectin protein and the P-selectin ligand protein to form a second binding mixture;

(d) measuring the amount of binding in the second binding mixture; and

(e) comparing the amount of binding in the first binding mixture with the amount of binding in the second binding mixture;

wherein the compound is capable of inhibiting P-selectin-mediated intercellular adhesion when a decrease in the
5 amount of binding of the second binding mixture occurs.

In another embodiment, the invention provides a method of identifying an inhibitor of E-selectin-mediated intercellular adhesion which comprises:

10 (a) combining a E-selectin protein with a P-selectin ligand protein comprising an amino acid sequence selected from the group consisting of the amino acid sequence set forth in SEQ ID NO:2 from amino acid 1 to amino acid 402, the amino acid sequence set forth in SEQ ID NO:2 from amino
15 acid 42 to amino acid 402, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 310, and the amino acid sequence set forth in SEQ ID NO:4, said combination forming a first binding mixture;

(b) measuring the amount of binding between the E-selectin protein and the P-selectin ligand protein in the
20 first binding mixture;

(c) combining a compound with the E-selectin protein and the P-selectin ligand protein to form a second binding mixture;

25 (d) measuring the amount of binding in the second binding mixture; and

(e) comparing the amount of binding in the first binding mixture with the amount of binding in the second binding mixture;

30 wherein the compound is capable of inhibiting E-selectin-mediated intercellular adhesion when a decrease in the amount of binding of the second binding mixture occurs.

The invention also encompasses processes for producing P-selectin ligand proteins which comprise (a) co-
35 transforming a host cell with a DNA encoding a P-selectin ligand protein and a DNA encoding a fucosyltransferase capable of synthesizing sialyl Lewis X (sLe^x) or sialyl

Lewis A (sLe^x) (such as an (α 1,3/ α 1,4) fucosyltransferase or an (α 1, 3) fucosyltransferase), each of said DNAs being operably linked to an expression control sequence; (b) culturing the host cell in suitable culture medium; and (c) purifying the P-selectin ligand protein from the culture medium. In certain other embodiments, the host cell is also co-transformed with a DNA encoding a paired basic amino acid converting enzyme and/or a DNA encoding a GlcNAc transferase (preferably a "core2 transferase"). In preferred embodiments, the P-selectin ligand protein is a full-length or soluble form.

In other embodiments, the present invention includes a P-selectin ligand protein having P-selectin ligand protein activity. In preferred embodiments, the ligand protein is a protein comprising the sequence from amino acid 42 to amino acid 60 of SEQ ID NO: 2, consisting essentially of the sequence from amino acid 42 to amino acid 60 of SEQ ID NO: 2, comprising the sequence from amino acid 42 to amino acid 88 of SEQ ID NO: 2, consisting essentially of the sequence from amino acid 42 to amino acid 88 of SEQ ID NO: 2, consisting essentially of the sequence from amino acid 42 to amino acid 118 of SEQ ID NO: 2, or consisting essentially of the sequence from amino acid 42 to amino acid 189 of SEQ ID NO: 2. In other preferred embodiments, at least one of the asparagine residues at positions 65, 111 and 292 of SEQ ID NO: 2 have been deleted or replaced. Certain preferred embodiments of the ligand protein comprises at least one of the tyrosine residues at positions 46, 48 and 51 of SEQ ID NO: 2. DNAs encoding these P-selectin ligand proteins, host cells transformed with such DNAs, process for producing protein by culturing such host cells, pharmaceutical compositions comprising the proteins, methods of identifying selectin binding inhibitors using the proteins, antibodies to the proteins and methods of inhibiting selectin mediated binding using the proteins are also encompassed by the invention.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a graph comparing the binding of P-selectin ligand proteins expressed with and without core2.

Fig. 2 is an autoradiograph of immunoprecipitations of
5 P-selectin ligand protein expressed with and without core2.

Fig. 3 depicts the results of flow cytometry analysis of the binding of P-selectin ligand protein (expressed with and without core2) to P-selectin/IgG chimera (LEC-γ1) and anti-P-selectin ligand protein monoclonal antibody (MAB
10 275).

Fig. 4 is an autoradiograph of proteins, including P-selectin ligand protein, which bound to P- and E-selectin/IgG chimeras.

Fig. 5 is a schematic representation of structural
15 features of the full length P-selectin ligand protein of SEQ ID NO: 2.

Fig. 6 is a schematic representation of several P-selectin ligand protein fragments constructed for the purpose of examining the role of N-linked glycosylation
20 sites in binding of the P-selectin ligand proteins to selectins.

Fig. 7 depicts the results of experiments to determine the role of N-linked glycosylation sites in binding of the P-selectin ligand proteins to selectins.

Fig. 8 is a schematic representation of several P-selectin ligand protein fragments constructed for the purpose of examining the role of sulfated tyrosine residues in binding of the P-selectin ligand proteins to selectins.

Figs. 9-11 depicts the results of experiments to
30 determine the role of sulfated tyrosine residues in binding of the P-selectin ligand proteins to selectins.

Fig. 12 is a schematic representation of several P-selectin ligand protein fragments constructed for the purpose of examining the effects of various deletions on
35 the binding of the P-selectin ligand proteins to selectins.

Fig. 13 is a schematic depiction of the quantitative plate binding assay of Example 4(c).

Figs. 14-17 depict the results of experiments comparing the binding of various deleted and altered P-selectin ligand proteins to selectins.

5 Fig. 18 is a schematic representation of several P-selectin ligand protein fragments constructed for the purpose of examining the effects of alteration of tyrosine residues in the anionic region of the P-selectin ligand proteins on selectin binding.

10 Figs. 19-21 depict the results of experiments comparing the binding of various deleted and altered P-selectin ligand proteins to selectins.

Fig. 22 depicts a proposed model for binding of P-selectin ligand proteins to P- and E-selectin.

15 Figs. 23 and 24 depict the results of experiments comparing the binding of various deleted and altered P-selectin ligand proteins to selectins.

Some of the foregoing figures employ a convention for numbering amino acids within the depicted constructs which is different than the residue numbering employed in SEQ ID NO:2. In the figures, residues are numbered using the first amino acid of soluble mature P-selectin ligand as a starting point. Hence, the residue numbers used in the figures are 41 less than those of SEQ ID NO:2. For example, residue 19 in the figures corresponds to residue 25 60 in SEQ ID NO:2.

Fig. 25 is an analysis of the expression products of CHO cells, already expressing 3/4 fucosyltransferase and Core2 transferase, which were transfected with psPSL.T7, ATM, I316 or psPSL.QC and amplified using methotrexate. Conditioned media was either analyzed directly or first 30 precipitated with LEC-γ1 and then analyzed by SDS-PAGE under non-reducing and reducing conditions.

Fig. 26. SDS-PAGE separation of myeloid cell membrane proteins affinity captured by P- and E-selectin. Membrane 35 lysates were prepared from U937 cells metabolically labeled with ³H-glucosamine and subjected to affinity precipitation with immobilized P- and E-selectin and control human IgG₁.

Eluted proteins were treated with ("reduced") or without ("non-reduced") DTT prior to gel electrophoresis. Lanes: 1, affinity capture by human IgG₁; 2, affinity capture by P-selectin; 3, affinity capture by E-selectin.

5 Fig. 27. Sequential affinity capture experiments. ³H-labeled U937 lysate species were affinity captured by P-or E-selectin, eluted, and then subjected to immunoprecipitation with anti-PSGL-1 antiserum Rb3443. Lanes: 1 and 2, control immunoprecipitations of fresh
10 myeloid cell membrane lysates using pre-immune rabbit serum (lane 1) and Rb3443 (lane 2); 3-5, immunoprecipitation with Rb3443 of myeloid cell membrane lysates previously affinity captured and eluted from P-selectin (lane 3), E-selectin (lane 4), and human IgG₁ (lane 5).

15 Fig. 28. Comparison of CD43 and PSGL-1 content of myeloid cell membrane extracts. Labeled U937 cell extracts were immunoprecipitated with anti-PSGL-1 rabbit polyclonal antibody Rb3443 or an anti-CD43 mouse MAb and then subjected to SDS-PAGE/autoradiography. Lanes: 1,
20 immunoprecipitation with control pre-immune rabbit serum; 2, immunoprecipitation with Rb3443; 3, immunoprecipitation with control isotype-matched mouse antibody; 4, immunoprecipitation with anti-CD43 antibody.

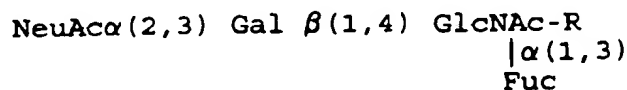
Fig. 29. COS transfection experiments. COS M6 cells
25 transfected with plasmids encoding PSGL-1 or CD43 as well as Fuc-TIIII or Fuc-TVII were metabolically labeled with ³⁵S-methionine, and membranes were prepared for affinity capture experiments as described in Materials and Methods. The cDNAs employed in the transfections are indicated above
30 the lanes. Precipitations were performed using (A) E-selectin, (B) P-selectin, and (C) anti-PSGL-1 antiserum Rb3443 and anti-CD43 MAb.

Fig. 30 summarizes the results of screening of various
P-selecint ligand proteins for inhibition of P- and E-
35 selectin binding (see Example 13).

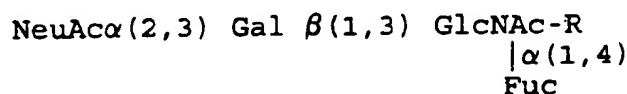
DETAILED DESCRIPTION OF THE INVENTION

The present inventors have for the first time identified and isolated a novel DNA which encodes a protein which acts as a ligand for P-selectin on human endothelial cells and platelets. The sequence of the DNA is set forth in SEQ ID NO:1. The complete amino acid sequence of the P-selectin ligand protein (i.e., the mature peptide plus the leader sequence) is characterized by the amino acid sequence set forth in SEQ ID NO:2 from amino acid 1 to amino acid 402. Hydrophobicity analysis and comparison with known cleavage patterns predict a signal sequence of 20 to 22 amino acids, i.e., amino acids 1 to 20 or amino acids 1 to 22 of SEQ ID NO:2. The P-selectin ligand protein contains a PACE (paired basic amino acid converting enzyme) cleavage site (-Arg-Asp-Arg-Arg-) at amino acids 38-41 of SEQ ID NO:2. The mature P-selectin ligand protein of the present invention is characterized by the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 402. A soluble form of the P-selectin ligand protein is characterized by containing amino acids 21 to 310 of SEQ ID NO:2. Another soluble form of the mature P-selectin ligand protein is characterized by the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 310. The soluble form of the P-selectin ligand protein is further characterized by being soluble in aqueous solution at room temperature. Of course, the corresponding DNA sequences as set forth in SEQ ID NO:1 encoding these proteins are also included in the subject invention.

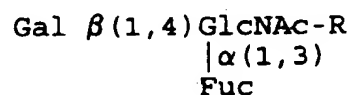
The P-selectin ligand of the invention is a glycoprotein which may contain one or more of the following terminal carbohydrates:



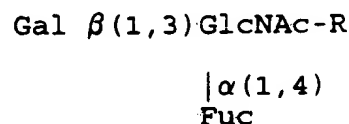
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where R= the remainder of the carbohydrate chain, which is covalently attached either directly to the P-selectin ligand protein or to a lipid moiety which is covalently attached to the P-selectin ligand protein. The P-selectin ligand glycoprotein of the invention may additionally be sulfated or otherwise post-translationally modified. As expressed in COS and CHO cells, full length P-selectin ligand protein (amino acids 1 to 402 of SEQ ID NO:2 or amino acids 42 to 402 of SEQ ID NO:2) is a homodimeric protein having an apparent molecular of 220 kD as shown by non-reducing SDS-polyacrylamide gel electrophoresis.

The structure of the full-length P-selectin ligand protein is schematically represented in Fig. 5. Three regions of the P-selectin ligand protein of SEQ ID NO:2 are: an extracellular domain (from about amino acid 21 to 310 of SEQ ID NO:2), a transmembrane domain (from about amino acid 311 to 332 of SEQ ID NO:2), and an intracellular, cytoplasmic domain (from about amino acid 333 to 402 of SEQ ID NO:2). The extracellular domain contains three consensus tripeptide sites (Asn-X-Ser/Thr) of potential N-linked glycosylation beginning at Asn residues 65, 111, and 292. The extracellular domain further contains three potential sites of tyrosine

sulfation at residues 46, 48, and 51. The region comprised of residues 55-267 contains a high percentage of proline, serine, and threonine including a subdomain of fifteen decameric repeats of the ten amino acid consensus sequence
5 Ala-Thr/Met-Glu-Ala-Gln-Thr-Thr-X-Pro/Leu-Ala/Thr, wherein X can be either Pro, Ala, Gln, Glu, or Arg. Regions such as these are characteristic of highly O-glycosylated proteins.

COS or CHO cells co-transfected with a gene encoding
10 the P-selectin ligand protein and a gene encoding fucosyltransferase (hereinafter FT), preferably an (α 1,3/ α 1,4) fucosyltransferase ("3/4FT"), are capable of binding to CHO cells expressing P-selectin on their surface, but are not capable of binding to CHO cells which
15 do not express P-selectin on their surface. In order to bind to P-selectin, either in purified form or expressed on the surface of CHO cells, the gene encoding the P-selectin ligand protein must be co-transfected with the gene encoding an FT, since transfection of either gene in the
20 absence of the other either abolishes or substantially reduces the P-selectin binding activity. The binding of the P-selectin ligand protein of the invention to P-selectin can be inhibited by EDTA or by a neutralizing monoclonal antibody specific for P-selectin. The binding
25 of the P-selectin ligand protein of the invention to P-selectin is not inhibited by a non-neutralizing monoclonal antibody specific for P-selectin or by an isotype control. These results characterize the binding specificity of the P-selectin ligand protein of the invention.

30 For the purposes of the present invention, a protein is defined as having "P-selectin ligand protein activity", i.e., variably referred to herein as a "P-selectin ligand protein", or as a "P-selectin ligand glycoprotein" or simply as a "P-selectin ligand", when it binds in a
35 calcium-dependent manner to P-selectin which is present on the surface of cells as in the CHO-P-selectin binding assay of Example 4, or to P-selectin which is affixed to another

surface, for example, as the chimeric P-selectin-IgG γ 1 protein of Example 4 is affixed to Petri dishes.

The glycosylation state of the P-selectin ligand protein of the invention was studied using a chimeric, soluble form of the P-selectin ligand protein, described in detail in Example 5(C) and designated sPSL.T7. The sPSL.T7 protein produced from COS cells co-transfected with 3/4FT is extensively modified by post-translational glycosylation, as described in detail in Example 6(C). Thus, it is believed that both N- and O-linked oligosaccharide chains, at least some of which are sialated, are present on the P-selectin ligand protein of the invention.

The P-selectin ligand protein of the invention may also bind to E-selectin. Conditioned medium from COS cells which have been co-transfected with the DNA encoding sPSL.T7 and with the DNA encoding 3/4FT, when coated on wells of plastic microtiter plates, causes CHO cells which express E-selectin to bind to the plates; however CHO cells which do not express E-selectin do not bind to such plates. The binding of CHO cells which express E-selectin to microtiter plates coated with conditioned medium from COS cells which have been co-transfected with the DNA encoding sPSL.T7 and with the DNA encoding 3/4FT is abolished in the presence of EDTA or of a neutralizing antibody specific for E-selectin. Conditioned medium from COS cells transfected only with the sPSL.T7 DNA does not cause binding of CHO cells which express E-selectin when coated on wells of microtiter plates. For these reasons, the P-selectin ligand protein of the invention is believed to be useful as an inhibitor of E-selectin-mediated intercellular adhesion in addition to P-selectin-mediated intercellular adhesion.

Antibodies raised against COS-produced soluble P-selectin ligand protein are immunoreactive with the major HL-60 glycoprotein that specifically binds P-selectin as determined by affinity capture using an immobilized Fc chimera of P-selectin. U937 cells bear a similar

immunoreactive glycoprotein ligand. Thus, a single glycoprotein species is observed upon EDTA elution of immobilized P-selectin previously incubated with detergent extracts of ³H-glucosamine labeled U937 cells. This major species exhibits an apparent molecular weight by SDS-PAGE of 220 kD under non-reducing conditions and 100 kD under reducing conditions. As with the comparable species isolated from HL-60 cells, this U937 ligand is immunoreactive with a polyclonal antibody raised against COS recombinant P-selectin ligand protein. In addition, affinity capture of E-selectin ligands from U937 cell and cell membrane preparations, using an immobilized Fc chimera of E-selectin, yield a single major species with identical mass and electrophoretic behavior as the major U937 P-selectin ligand. Thus, E- and P-selectin recognize the same major glycoprotein ligand in U937 cells, a glycoprotein ligand immunoreactive with an anti-P-selectin ligand protein antibody and possessing the same apparent mass and electrophoretic behavior as full length, recombinant P-selectin ligand protein.

Fragments of the P-selectin ligand protein which are capable of interacting with P-selectin or which are capable of inhibiting P-selectin-mediated intercellular adhesion are also encompassed by the present invention. Such fragments comprise amino acids 21 to 54 of SEQ ID NO:2, a region of the P-selectin ligand protein having a low frequency of serine and threonine residues; amino acids 55 to 127 of SEQ ID NO:2, having a high frequency of proline, serine, and threonine in addition to two consensus sequences for asparagine-linked glycosylation (Asn-X-Ser/Thr); another larger fragment, amino acids 128 to 267 of SEQ ID NO:2, having both a high frequency of proline, serine, and threonine and containing fifteen repeats of the following ten amino acid consensus sequence: Ala-(Thr/Met)-Glu-Ala-Gln-Thr-Thr-(Pro/Arg/Gln/Ala/Glu)-(Leu/Pro)-(Ala/Thr) (smaller fragments within this large fragment may also retain the capacity to interact with P-selectin or act

as inhibitors of P-selectin-mediated intercellular adhesion); the region containing a consensus sequence for asparagine-linked glycosylation and comprising amino acids 268 to 308 of SEQ ID NO:2; the hydrophobic region of the protein represented by amino acids 309 to 333 of SEQ ID NO:2; and the amphophilic region of the P-selectin ligand protein from amino acids 334 to 402 of SEQ ID NO:2. Additional fragments may comprise amino acid 43 to amino acid 56 of SEQ ID NO:2 or amino acid 42 to amino acid 60 of SEQ ID NO:2, with one or more sulfated or phosphorylated (Domcheck et al., Biochemistry 31:9865-9870 (1992)) tyrosines at amino acid 46, amino acid 48, and/or amino acid 51. Fragments of the P-selectin ligand protein may be in linear form or they may be cyclized using known methods, for example, as described in H.U. Saragovi, et al., Bio/Technology 10, 773-778 (1992) and in R.S. McDowell, et al., J. Amer. Chem. Soc. 114, 9245-9253 (1992), both of which are incorporated herein by reference. For the purposes of the present invention, all references to "P-selectin ligand protein" herein include fragments capable of binding to P-selectin.

Such fragments may be fused to carrier molecules such as immunoglobulins, to increase the valency of P-selectin ligand binding sites. For example, soluble forms of the P-selectin ligand protein such as the fragment from amino acid 42 to amino acid 295 of SEQ ID NO:2 may be fused through "linker" sequences to the Fc portion of an immunoglobulin. For a bivalent form of the P-selectin ligand protein, such a fusion could be to the Fc portion of an IgG molecule as in Example 5(D) and in SEQ ID NO:6. Other immunoglobulin isotypes may also be used to generate such fusions. For example, a P-selectin ligand protein - IgM fusion would generate a decavalent form of the P-selectin ligand protein of the invention.

As detailed in the Examples below, the P-selectin ligand protein of the invention was initially obtained using an expression cloning approach (Clark et al., U.S.

4,675,285). A cDNA library was constructed from the human promyelocytic cell line HL-60 (S.J. Collins, et al., Nature 270, 347-349 (1977), ATCC No. CCL 240). This library was cotransfected into COS cells with a DNA encoding a 3/4FT, and the cotransfectants were screened for binding to a chimeric molecule consisting of the extracellular portion of P-selectin and the Fc portion of a human IgG γ 1 monoclonal antibody. Cotransfectants which bound to the chimeric P-selectin were enriched for cDNAs encoding the P-selectin ligand protein. This screening process was repeated several times to enrich the plasmid population further for cDNAs encoding the P-selectin ligand protein. In a second cloning stage, the enriched plasmid population was again cotransfected into COS cells with the 3/4FT gene and screened for binding to a fluorescently labeled CHO cell line which expressed P-selectin on the cell surface. A single cDNA clone was obtained from this approach and was designated pMT21:PL85. The pMT21:PL85 plasmid was deposited with the American Type Culture Collection on October 16, 1992 and given the accession number ATCC 69096.

One novel DNA of the present invention is set forth in SEQ ID NO:1. The DNA of the present invention may encode a variety of forms of the P-selectin ligand protein. For example, in one embodiment, the DNA of the invention encodes the entire P-selectin ligand protein having the amino acid sequence set forth in SEQ ID NO:2 from amino acid 1 to amino acid 402. In another embodiment, the DNA of the invention encodes a form of the P-selectin ligand protein which lacks the signal sequence and which is characterized by the amino acid sequence set forth in SEQ ID NO:2 from amino acid 21 to amino acid 402. In yet another embodiment, the DNA of the invention encodes the mature P-selectin ligand protein characterized by the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 402. Another embodiment of the DNA of the invention encodes a soluble form of the P-selectin ligand protein characterized by the amino acid sequence set forth

in SEQ ID NO:2 from amino acid 1 to amino acid 310. The DNA of the invention is also embodied in a DNA encoding a soluble form of the mature P-selectin ligand protein, said protein being characterized by the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 310. The DNA of the invention is further embodied in a DNA sequence encoding a soluble form of the P-selectin ligand protein which lacks the signal sequence, said protein being characterized by the amino acid sequence set forth in SEQ ID NO:2 from amino acid 21 to amino acid 310. The DNA of the present invention is free from association with other human DNAs and is thus characterized as an isolated DNA. As detailed above, DNAs which encode P-selectin ligand fragments which interact with P-selectin are also included in the present invention.

The expression of P-selectin ligand protein mRNA transcripts has been observed in a variety of human cell lines (HL-60, THP-1, U937) and in human monocytes and polymorphonuclear leukocytes by Northern analysis using a P-selectin ligand protein cDNA probe. In all of these cell lines, a major transcript of 2.5 kb was observed. A minor species of approximately 4 kb was observed in the HL60 and U937 cell lines and in polymorphonuclear leukocytes. In contrast, no P-selectin ligand mRNA expression was detected in the human hepatoblastoma cell line HepG2.

The P-selectin ligand protein of the invention is encoded by a single copy gene and is not part of a multi-gene family, as determined by Southern blot analysis. The genomic form of the P-selectin ligand protein of the invention contains a large intron of approximately 9 kb located at nucleotide 54 in the 5' untranslated region. In polymorphonuclear leukocytes and monocytes, the P-selectin ligand protein of the invention is encoded by the DNA sequence set forth in SEQ ID NO:3. In this embodiment, the P-selectin ligand protein contains sixteen repeat regions. The isolated DNA of the invention is correspondingly also embodied in the DNA sequence set forth in SEQ ID NO:3 and

is contained on plasmid pPL85R16 which was deposited with the American Type Culture Collection on October 22, 1993 and given the Accession Number ATCC 75577.

The invention also encompasses allelic variations of the isolated DNA as set forth in SEQ ID NO:1 or of the isolated DNA as set forth in SEQ ID NO:3, that is, naturally-occurring alternative forms of the isolated DNA of SEQ ID NO: 1 or SEQ ID NO:3 which also encode proteins having P-selectin ligand activity. Also included in the invention are isolated DNAs which hybridize to the DNA set forth in SEQ ID NO:1 or to the DNA set forth in SEQ ID NO:3 under stringent (e.g. 4xSSC at 65°C or 50% formamide and 4xSSC at 42°C), or relaxed (4xSSC at 50°C or 30-40% formamide at 42°C) conditions, and which have P-selectin ligand protein activity. Isolated DNA sequences which encode the P-selectin ligand protein but which differ from the DNA set forth in SEQ ID NO:1 or from the DNA set forth in SEQ ID NO:3 by virtue of the degeneracy of the genetic code and which have P-selectin ligand protein activity are also encompassed by the present invention. Variations in the DNA as set forth in SEQ ID NO:1 or in the DNA as set forth in SEQ ID NO:3 which are caused by point mutations or by induced modifications which enhance the P-selectin ligand activity, half-life or production level are also included in the invention. For the purposes of the present invention all references herein to the "DNA of SEQ ID NO:1" include, in addition to DNAs comprising the specific DNA sequence set forth in SEQ ID NO:1, DNAs encoding the mature P-selectin ligand protein of SEQ ID NO:2; DNAs encoding fragments of the P-selectin ligand protein of SEQ ID NO:2 which are capable of binding to P-selectin; DNAs encoding soluble forms of the P-selectin ligand protein of SEQ ID NO:2; allelic variations of the DNA sequence of SEQ ID NO:1; DNAs which hybridize to the DNA sequence of SEQ ID NO:1 and which encode proteins having P-selectin ligand protein activity; DNAs which differ from the DNA of SEQ ID NO:1 by virtue of degeneracy of the genetic code; and the

variations of the DNA sequence of SEQ ID NO:1 set forth above. Similarly, all references to the "DNA of SEQ ID NO:3" include in addition to the specific sequence set forth in SEQ ID NO:3, DNAs encoding the mature P-selectin ligand protein of SEQ ID NO:4; DNAs encoding fragments of the P-selectin ligand protein of SEQ ID NO:4 which are capable of binding to P-selectin; DNAs encoding soluble forms of the P-selectin ligand protein of SEQ ID NO:4; allelic variations of the DNA of SEQ ID NO:3; DNAs which hybridize to the DNA sequence of SEQ ID NO:3 and which encode proteins having P-selectin ligand protein activity; DNAs which differ from the DNA of SEQ ID NO:3 by virtue of degeneracy of the genetic code; and the variations of the DNA of SEQ ID NO:3 set forth above.

15 A DNA encoding a soluble form of the P-selectin ligand protein may be prepared by expression of a modified DNA in which the regions encoding the transmembrane and cytoplasmic domains of the P-selectin ligand protein are deleted and/or a stop codon is introduced 3' to the codon for the amino acid at the carboxy terminus of the extracellular domain. For example, hydrophobicity analysis predicts that the P-selectin ligand protein set forth in SEQ ID NO:2 has a transmembrane domain comprised of amino acids 311 to 332 of SEQ ID NO:2 and a cytoplasmic domain comprised of amino acids 333 to 402 of SEQ ID NO:2. A modified DNA as described above may be made by standard molecular biology techniques, including site-directed mutagenesis methods which are known in the art or by the polymerase chain reaction using appropriate oligonucleotide primers. Methods for producing several DNAs encoding various soluble P-selectin ligand proteins are set forth in Example 5.

35 A DNA encoding other fragments and altered forms of P-selectin ligand protein may be prepared by expression of modified DNAs in which portions of the full-length sequence have been deleted or altered. Substantial deletions of the P-selectin ligand protein sequence can be made while

retaining P-selectin ligand protein activity. For example, P-selectin ligand proteins comprising the sequence from amino acid 42 to amino acid 189 of SEQ ID NO: 2, the sequence from amino acid 42 to amino acid 118 of SEQ ID NO: 2, or the sequence from amino acid 42 to amino acid 89 of SEQ ID NO: 2 each retain the P-selectin protein binding activity and the ability to bind to E-selectin. P-selectin ligand proteins in which one or more N-linked glycosylation sites (such as those at amino acids 65, 111 and 292 of SEQ ID NO: 2) have been changed to other amino acids or deleted also retain P-selectin protein binding activity and the ability to bind E-selectin. P-selectin ligand proteins comprising from amino acid 42 to amino acid 60 of SEQ ID NO:2 (which includes a highly anionic region of the protein from amino acid 45 to amino acid 58 of SEQ ID NO:2) also retain P-selectin ligand protein activity; however, P-selectin ligand proteins limited to such sequence do not bind to E-selectin. Preferably, a P-selectin ligand protein retains at least one (more preferably at least two and most preferably all three) of the tyrosine residues found at amino acids 46, 48 and 51 of SEQ ID NO: 2, sulfation of which may contribute to P-selectin ligand protein activity. Construction of DNAs encoding these and other active fragments or altered forms of P-selectin ligand protein may be accomplished in accordance with methods known to those skilled in the art.

The isolated DNA of the invention may be operably linked to an expression control sequence such as the pMT2 or pED expression vectors disclosed in Kaufman et al., Nucleic Acids Res. 19, 4485-4490 (1991), in order to produce the P-selectin ligand recombinantly. Many suitable expression control sequences are known in the art. General methods of expressing recombinant proteins are also known and are exemplified in R. Kaufman, Methods in Enzymology 185, 537-566 (1990). As defined herein "operably linked" means enzymatically or chemically ligated to form a covalent bond between the isolated DNA of the invention and

the expression control sequence, in such a way that the P-selectin ligand protein is expressed by a host cell which has been transformed (transfected) with the ligated DNA/expression control sequence.

5 Several endoproteolytic enzymes are known which cleave precursor peptides at the carboxyl side of paired amino acid sequences (e.g., -Lys-Arg- and -Arg-Arg-) to yield mature proteins. Such enzymes are generally known as paired basic amino acid converting enzymes or PACE, and
10 their use in recombinant production of mature peptides is extensively disclosed in WO 92/09698 and U.S. Application Serial No. 07/885,972, both of which are incorporated herein by reference. The PACE family of enzymes are known
15 to increase the efficiency of proteolytic processing of precursor polypeptides in recombinant host cells. As mentioned above, the P-selectin ligand protein of the invention contains such a PACE cleavage site.

 The soluble mature P-selectin ligand protein of the present invention may be made by a host cell which contains
20 a DNA sequence encoding any soluble P-selectin ligand protein as described herein and a DNA sequence encoding PACE as described in WO 92/09698 and U.S. Application Serial No. 07/885,972, incorporated herein by reference, or using the DNA sequence of SEQ ID NO:5. Such a host cell
25 may contain the DNAs as the result of co-transformation or sequential transformation of separate expression vectors containing the soluble P-selectin ligand protein DNA and the PACE DNA, respectively. A third DNA which encodes a 3/4FT may also be co-transformed with the DNAs encoding the
30 P-selectin ligand protein and PACE. Alternatively, the host cell may contain the DNAs as the result of transformation of a single expression vector containing both soluble P-selectin ligand protein DNA and PACE DNA. Construction of such expression vectors is within the level
35 of ordinary skill in molecular biology. Methods for co-transformation and transformation are also known.

Many DNA sequences encoding PACE are known. For example, a DNA encoding one form of PACE, known as furin, is disclosed in A.M.W. van den Ouweland et al., Nucl. Acids Res. 18, 664 (1990), incorporated herein by reference. A
5 cDNA encoding a soluble form of PACE, known as PACESOL, is set forth in SEQ ID NO:5. DNAs encoding other forms of PACE also exist, and any such PACE-encoding DNA may be used to produce the soluble mature P-selectin ligand protein of the invention, so long as the PACE is capable of cleaving
10 the P-selectin ligand protein at amino acids 38-41. Preferably, a DNA encoding a soluble form of PACE is used to produce the soluble mature P-selectin ligand protein of the present invention.

The DNAs encoding a soluble form of the P-selectin
15 ligand protein and PACE, separately or together, may be operably linked to an expression control sequence such as those contained in the pMT2 or pED expression vectors discussed above, in order to produce the PACE-cleaved soluble P-selectin ligand recombinantly. Additional
20 suitable expression control sequences are known in the art. Examples 3(C) and 3(D) below set forth methods for producing the soluble mature P-selectin ligand protein of the invention.

A number of types of cells may act as suitable host
25 cells for expression of the P-selectin ligand protein. Suitable host cells are capable of attaching carbohydrate side chains characteristic of functional P-selectin ligand protein. Such capability may arise by virtue of the presence of a suitable glycosylating enzyme within the host
30 cell, whether naturally occurring, induced by chemical mutagenesis, or through transfection of the host cell with a suitable expression plasmid containing a DNA sequence encoding the glycosylating enzyme. Host cells include, for example, monkey COS cells, Chinese Hamster Ovary (CHO)
35 cells, human kidney 293 cells, human epidermal A431 cells, human Colo205 cells, 3T3 cells, CV-1 cells, other transformed primate cell lines, normal diploid cells, cell

strains derived from in vitro culture of primary tissue, primary explants, HeLa cells, mouse L cells, BHK, HL-60, U937, or HaK cells.

5 The P-selectin ligand protein may also be produced by operably linking the isolated DNA of the invention and one or more DNAs encoding suitable glycosylating enzymes to suitable control sequences in one or more insect expression vectors, and employing an insect expression system. Materials and methods for baculovirus/insect cell
10 expression systems are commercially available in kit form from, e.g., Invitrogen, San Diego, California, U.S.A. (the MaxBac® kit), and such methods are well known in the art, as described in Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987), incorporated
15 herein by reference. Soluble forms of the P-selectin ligand protein may also be produced in insect cells using appropriate isolated DNAs as described above. A DNA encoding a form of PACE may further be co-expressed in an insect host cell to produce a PACE-cleaved form of the P-selectin ligand protein.
20

Alternatively, it may be possible to produce the P-selectin ligand protein in lower eukaryotes such as yeast or in prokaryotes such as bacteria. Potentially suitable yeast strains include *Saccharomyces cerevisiae*,
25 *Schizosaccharomyces pombe*, *Kluyveromyces* strains, *Candida*, or any yeast strain capable of expressing heterologous proteins. Potentially suitable bacterial strains include *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, or any bacterial strain capable of expressing
30 heterologous proteins. If the P-selectin ligand protein is made in yeast or bacteria, it is necessary to attach the appropriate carbohydrates to the appropriate sites on the protein moiety covalently, in order to obtain the glycosylated P-selectin ligand protein. Such covalent
35 attachments may be accomplished using known chemical or enzymatic methods.

The P-selectin ligand protein of the invention may also be expressed as a product of transgenic animals, e.g., as a component of the milk of transgenic cows, goats, pigs, or sheep which are characterized by somatic or germ cells containing a DNA sequence encoding the P-selectin ligand protein.

The P-selectin binding activity of a P-selectin protein may be enhanced by co-transformation of a host cell with a GlcNAc transferase, preferably UDP-GlcNAc:Gal β 1-3GalNAc-R(GlcNAc to GalNAc) β 1-6 GlcNAc transferase (EC 2.4.1.102), also known as "core2 transferase."

O-linked glycans present on P-selectin ligand protein have been shown to be important for binding to P-selectin (D. Sako et al., Cell 75, 1179-1186 (1993)). It has been reported that sialyl Le^x on O-linked glycans of myeloid cells are presented on complex, branched structures (Maemura, K. and Fukuda, M., J. Biol. Chem. 267, 24379-24386 (1992)). The enzyme responsible for generating such oligosaccharide structures is "core2". The core2 enzyme activity is found at very low levels in COS cells and at trace levels in CHO cells. Host cells co-transformed with DNAs encoding a P-selectin ligand protein, an (α 1,3/ α 1,4) fucosyltransferase and core2 can produce P-selectin ligand protein exhibiting 20-30 fold enhanced binding to P-selectin.

In certain preferred embodiments, P-selectin ligand protein is produced by co-transfecting a host cell with DNAs encoding soluble P-selectin ligand protein, 3/4FT, core2 and PACE.

The P-selectin ligand protein of the invention may be prepared by culturing transformed host cells under culture conditions necessary to express a P-selectin binding glycoprotein. The resulting expressed glycoprotein may then be purified from culture medium or cell extracts. Soluble forms of the P-selectin ligand protein of the invention can be purified by affinity chromatography over Lentil lectin-Sepharose® and subsequent elution with 0.5M

α -methyl-mannoside. The eluted soluble P-selectin ligand protein can then be further purified and concentrated by a 0-70% ammonium sulfate precipitation step. The protein is then recovered, resuspended, and further purified by size exclusion chromatography over a TSK G4000SW_{XL}. Alternatively, full length forms of the P-selectin ligand protein of the invention can be purified by preparing a total membrane fraction from the expressing cell and extracting the membranes with a non-ionic detergent such as Triton X-100. The detergent extract can then be passed over an affinity column comprised of immobilized P-selectin, and the P-selectin ligand protein can be eluted from the column with 10mM EDTA in a buffer containing 0.1% detergent. The material eluted from the affinity column can then be dialyzed to remove EDTA and further purified over a Lentil lectin-Sepharose® affinity column, again eluting with 0.5M α -methyl-mannoside.

Alternatively, the P-selectin ligand protein of the invention is concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred (e.g., S-Sepharose® columns). The purification of the P-selectin ligand protein from culture supernatant may also include one or more column steps over such affinity resins as concanavalin A-agarose, heparin-toyopearl® or Cibacrom blue 3GA Sepharose®; or by hydrophobic interaction chromatography using such resins as

phenyl ether, butyl ether, or propyl ether; or by immunoaffinity chromatography.

Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify the P-selectin ligand protein. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a substantially homogeneous isolated recombinant protein. The P-selectin ligand protein thus purified is substantially free of other mammalian proteins and is defined in accordance with the present invention as "isolated P-selectin ligand protein".

Isolated P-selectin ligand protein may be useful in treating conditions characterized by P- or E-selectin mediated intercellular adhesion. Such conditions include, without limitation, myocardial infarction, bacterial or viral infection, metastatic conditions, inflammatory disorders such as arthritis, acute respiratory distress syndrome, asthma, emphysema, delayed type hypersensitivity reaction, systemic lupus erythematosus, thermal injury such as burns or frostbite, autoimmune thyroiditis, experimental allergic encephalomyelitis, multiple sclerosis, multiple organ injury syndrome secondary to trauma, diabetes, Reynaud's syndrome, neutrophilic dermatosis (Sweet's syndrome), inflammatory bowel disease, Grave's disease, glomerulonephritis, gingivitis, periodontitis, hemolytic uremic syndrome, ulcerative colitis, Crohn's disease, necrotizing enterocolitis, granulocyte transfusion associated syndrome, cytokine-induced toxicity, and the like. Isolated P-selectin ligand protein may also be useful in organ transplantation, both to prepare organs for transplantation and to quell organ transplant rejection. Isolated P-selectin ligand protein may be used to treat hemodialysis and leukopheresis patients. Additionally, isolated P-selectin ligand protein may be used as an antimetastatic agent. Isolated P-selectin ligand protein

may be used itself as an inhibitor of P- or E-selectin-mediated intercellular adhesion or to design inhibitors of P- or E-selectin-mediated intercellular adhesion. The present invention encompasses both pharmaceutical compositions containing isolated P-selectin ligand protein and therapeutic methods of treatment or use which employ isolated P-selectin ligand protein.

Isolated P-selectin ligand protein, purified from cells or recombinantly produced, may be used as a pharmaceutical composition when combined with a pharmaceutically acceptable carrier. Such a composition may contain, in addition to P-selectin ligand protein and carrier, diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The characteristics of the carrier will depend on the route of administration. The pharmaceutical composition of the invention may also contain cytokines, lymphokines, or other hematopoietic factors such as M-CSF, GM-CSF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, G-CSF, Meg-CSF, stem cell factor, and erythropoietin. The pharmaceutical composition may contain thrombolytic or anti-thrombotic factors such as plasminogen activator and Factor VIII. The pharmaceutical composition may further contain other anti-inflammatory agents. Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect with isolated P-selectin ligand protein, or to minimize side effects caused by the isolated P-selectin ligand protein. Conversely, isolated P-selectin ligand protein may be included in formulations of the particular cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent to minimize side effects of the cytokine, lymphokine, other

hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent.

The pharmaceutical composition of the invention may be in the form of a liposome in which isolated P-selectin ligand protein is combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as micelles, insoluble monolayers, liquid crystals, or lamellar layers which in aqueous solution. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Patent No. 4,235,871; U.S. Patent No. 4,501,728; U.S. Patent No. 4,837,028; and U.S. Patent No. 4,737,323, all of which are incorporated herein by reference.

As used herein, the term "therapeutically effective amount" means the total amount of each active component of the pharmaceutical composition or method that is sufficient to show a meaningful patient benefit, i.e., healing of chronic conditions characterized by P-selectin- or E-selectin-mediated cellular adhesion or increase in rate of healing of such conditions. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

In practicing the method of treatment or use of the present invention, a therapeutically effective amount of isolated P-selectin ligand protein is administered to a mammal having a P-selectin-mediated disease state. Isolated P-selectin ligand protein may be administered in accordance with the method of the invention either alone or in combination with other therapies such as treatments employing cytokines, lymphokines or other hematopoietic

factors. When co-administered with one or more cytokines, lymphokines or other hematopoietic factors, isolated P-selectin ligand protein may be administered either simultaneously with the cytokine(s), lymphokine(s), other
5 hematopoietic factor(s), thrombolytic or anti-thrombotic factors, or sequentially. If administered sequentially, the attending physician will decide on the appropriate sequence of administering isolated P-selectin ligand protein in combination with cytokine(s), lymphokine(s),
10 other hematopoietic factor(s), thrombolytic or anti-thrombotic factors.

Administration of isolated P-selectin ligand protein used in the pharmaceutical composition or to practice the method of the present invention can be carried out in a
15 variety of conventional ways, such as oral ingestion, inhalation, or cutaneous, subcutaneous, or intravenous injection. Intravenous administration to the patient is preferred.

When a therapeutically effective amount of isolated P-selectin ligand protein is administered orally, isolated P-selectin ligand protein will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier such as
25 a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% isolated P-selectin ligand protein, and preferably from about 25 to 90% isolated P-selectin ligand protein. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal
30 or plant origin such as peanut oil, mineral oil, soybean oil, or sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol,
35 propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to 90% by weight of isolated P-selectin

ligand protein and preferably from about 1 to 50% isolated P-selectin ligand protein.

When a therapeutically effective amount of isolated P-selectin ligand protein is administered by intravenous, cutaneous or subcutaneous injection, isolated P-selectin ligand protein will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable protein solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection should contain, in addition to isolated P-selectin ligand protein an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additive known to those of skill in the art.

The amount of isolated P-selectin ligand protein in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of isolated P-selectin ligand protein with which to treat each individual patient. Initially, the attending physician will administer low doses of isolated P-selectin ligand protein and observe the patient's response. Larger doses of isolated P-selectin ligand protein may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not increased further. It is contemplated that the various pharmaceutical compositions used to practice the method of the present invention should contain about 0.1 μ g to about 100 mg of isolated P-selectin ligand protein per kg body weight.

The duration of intravenous therapy using the pharmaceutical composition of the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. It is contemplated that the duration of each application of the isolated P-selectin ligand protein will be in the range of 12 to 24 hours of continuous intravenous administration. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

Isolated P-selectin ligand protein of the invention may also be used to immunize animals to obtain polyclonal and monoclonal antibodies which specifically react with the P-selectin ligand protein and which may inhibit P-selectin-mediated cellular adhesion. Such antibodies may be obtained using the entire P-selectin ligand protein as an immunogen, or by using fragments of P-selectin ligand protein such as the soluble mature P-selectin ligand protein. Smaller fragments of the P-selectin ligand protein may also be used to immunize animals, such as the fragments set forth below: amino acid 42 to amino acid 56 of SEQ ID NO:2 and amino acid 127 to amino acid 138 of SEQ ID NO:2. An additional peptide immunogen comprises amino acid 238 to amino acid 248 of SEQ ID NO:2, with an alanine residue added to the amino terminus of the peptide. Another peptide immunogen comprises amino acid 43 to amino acid 56 of SEQ ID NO:2 having a sulfated tyrosine in any or all of positions 46, 48 or 51. The peptide immunogens additionally may contain a cysteine residue at the carboxyl terminus, and are conjugated to a hapten such as keyhole limpet hemocyanin (KLH). Additional peptide immunogens may be generated by replacing tyrosine residues with sulfated tyrosine residues. Methods for synthesizing such peptides are known in the art, for example, as in R.P. Merrifield, J.Amer.Chem.Soc. 85, 2149-2154 (1963); J.L. Krstenansky, et al., FEBS Lett. 211, 10 (1987).

Monoclonal antibodies binding to P-selectin ligand glycoprotein or to complex carbohydrate moieties characteristic of the P-selectin ligand glycoprotein may be useful diagnostic agents for the immunodetection of inflammatory diseases and some forms of cancer. Some cancerous cells, such as small cell lung carcinomas, may express detectable levels of the P-selectin ligand protein. This abnormal expression of the P-selectin ligand protein by cancer cells may play a role in the metastasis of these cells.

Neutralizing monoclonal antibodies binding to P-selectin ligand glycoprotein or to complex carbohydrates characteristic of P-selectin ligand glycoprotein may also be useful therapeutics for both inflammatory diseases and also in the treatment of some forms of cancer where abnormal expression of P-selectin ligand protein is involved. These neutralizing monoclonal antibodies are capable of blocking the selectin mediated intercellular adherence function of the P-selectin ligand protein. By blocking the binding of P-selectin ligand protein, the adherence of leukocytes to sites of inappropriate inflammation is either abolished or markedly reduced. In the case of cancerous cells or leukemic cells, neutralizing monoclonal antibodies against P-selectin ligand protein may be useful in detecting and preventing the metastatic spread of the cancerous cells which may be mediated by the P-selectin ligand protein. In addition, the monoclonal antibodies bound to these cells may target the cancerous cells for antibody-dependent cell mediated cytotoxicity (ADCC), thus helping to eliminate the cancerous cells. Human antibodies which react with the P-selectin ligand protein may be produced in transgenic animals which contain human immunoglobulin encoding genes in their germ lines. Example 7 below sets forth production of a rabbit polyclonal antibody specific P-selectin ligand protein fragments.

P-selectin ligand protein of the invention may also be used to screen for agents which are capable of binding to P-selectin ligand protein and thus may act as inhibitors of P-selectin- or E-selectin-mediated intercellular adhesion.

5 Binding assays using a desired binding protein, immobilized or not, are well known in the art and may be used for this purpose using the P-selectin ligand protein of the invention. Appropriate screening assays may be cell-based, as in Examples 3 and 9 below. Alternatively, purified

10 protein based screening assays may be used to identify such agents. For example, P-selectin ligand protein may be immobilized in purified form on a carrier and binding to purified P-selectin may be measured in the presence and in the absence of potential inhibiting agents. A suitable

15 binding assay may alternatively employ purified P-selectin immobilized on a carrier, with a soluble form of P-selectin ligand protein of the invention.

Any P-selectin ligand protein may be used in the screening assays described above. For example, the full-

20 length P-selectin ligand protein set forth in SEQ ID NO:2 from amino acid 1 to amino acid 402 may be used to screen for inhibitors; or the mature P-selectin ligand protein set forth in SEQ ID NO:2 from amino acid 42 to amino acid 402 may be used to screen for inhibitors, or the soluble mature

25 P-selectin ligand protein set forth in SEQ ID NO:2 from amino acid 42 to amino acid 310 may be used to screen for inhibitors. Alternatively, the P-selectin ligand protein of SEQ ID NO:4 from amino acid 1 to amino acid 412, or a mature form of the P-selectin ligand protein as set forth

30 in SEQ ID NO:4 from amino acid 42 to amino acid 412, or a soluble mature form of the P-selectin ligand protein set forth in SEQ ID NO:4 from amino acid 42 to amino acid 320 may be used to screen for inhibitors of intercellular adhesion in accordance with the present invention.

35 In such a screening assay, a first binding mixture is formed by combining P-selectin or E-selectin and P-selectin ligand protein, and the amount of binding in the first

binding mixture (B_0) is measured. A second binding mixture is also formed by combining P- or E-selectin, P-selectin ligand protein, and the compound or agent to be screened, and the amount of binding in the second binding mixture (B) is measured. The amounts of binding in the first and second binding mixtures are compared, for example, by performing a B/B_0 calculation. A compound or agent is considered to be capable of inhibiting P- or E-selectin mediated intercellular adhesion if a decrease in binding in the second binding mixture as compared to the first binding mixture is observed. The formulation and optimization of binding mixtures is within the level of skill in the art, such binding mixtures may also contain buffers and salts necessary to enhance or to optimize binding, and additional control assays may be included in the screening assay of the invention.

Compounds found to reduce by at least about 10%, preferably greater than about 50% or more of the binding activity of P-selectin ligand protein to P- or E-selectin may thus be identified and then secondarily screened in other selectin binding assays, including assays binding to L-selectin and *in vivo* assays. By these means compounds having inhibitory activity for selectin-mediated intercellular adhesion which may be suitable as anti-inflammatory agents may be identified.

EXAMPLE 1**CLONING OF THE P-SELECTIN LIGAND PROTEIN GENE****A. Construction of the HL60 cDNA library**

5 An HL60 cDNA library was constructed for expression cloning the P-selectin ligand. PolyA⁺ RNA was isolated from total RNA from the human promyelocytic cell line HL60 (S.J. Collins, et al., *supra*) using a Fast Track mRNA Isolation
10 Kit (Invitrogen; San Diego, CA). Double stranded cDNA was synthesized from the polyA⁺ RNA fraction and blunt-end ligated with EcoRI adaptors (5'-AATTCGTCGACTCTAGAG-3', SEQ ID NO:7; 5'-CTCTAGAGTCGACGG-3', SEQ ID NO:8). The cDNA was ligated into the expression vector pMT21 (R. Kaufman et
15 al., J. Mol. Cell. Biol. 9, 946-958 (1989) that had been incubated sequentially with EcoRI endonuclease and calf intestinal alkaline phosphatase and gel purified. The ligation product was electroporated in 2 μ l aliquots into competent *E. coli* DH5 α cells and grown in 1 ml of SOB
20 medium (J. Sambrook et al., Molecular Cloning: A Laboratory Manual, New York, Cold Spring Harbor Laboratory Press, p1.90 (1989)) which has been supplemented with 10 mM MgCl₂, 10 mM MgSO₄, and 2% glycerol for one hour at 37°C. In order to divide the library into smaller subsets, an
25 aliquot from each ml of bacterial suspension was plated onto agar plates in the presence of ampicillin, and the number of colonies per ml was calculated. Assuming that each colony represented one cDNA clone, 600,000 clones were generated and divided into subsets of approximately 16,000
30 clones per pool. Each of the 38 pools were grown overnight in L-broth in the presence of ampicillin and the plasmids were purified over a CsCl gradient.

B. Screening for the P-selectin ligand protein gene

35 In the first stage, the LEC- γ 1 binding assay of Example 4(A) was utilized to pan the HL60 cDNA library and thereby to enrich for the plasmid of interest. Six μ g of each HL60

cDNA library pool was co-transfected with 2 μ g of α 3/4FT gene (Example 2) into COS cells. Approximately 45 hours post-transfection, the COS cells were lifted from the plates by incubating the cells in 1 mM EGTA for 15 min. at 37°C, followed by scraping with cell lifters. The cells were washed twice in Hanks buffered saline solution containing 1 mM calcium (HBSS). The cells were resuspended in 4 ml of HBSS. The resuspended transfected COS cells were screened using the LEC- γ 1 binding assay described in Example 4(A).

The plasmids from adherent COS cells were recovered from a Hirts extract [B. Hirts, J. Mol. Biol., 26, 365-369 (1967)] and then electroporated into E. coli DH5 α cells for amplification. The enriched population of plasmids was purified over a CsCl gradient and re-transfected along with the 3/4FT gene (Example 2) into COS cells. The transfection, screening, and plasmid amplification process was repeated for a total of three times before a pool that bound to the LEC- γ 1-coated plates was visually detected. The positive plasmid pool was subsequently broken down into subsets. This involved electroporating the Hirts extract from the positive pool into E. coli DH5 α cells and quantitating colonies per ml as described above. Various pool sizes were produced by plating out a predetermined number of colonies on agar plates in the presence of ampicillin. Duplicate plates were prepared by performing nitrocellulose lifts and storing the filters on new agar plates. The duplicate plates served as reference plates for selecting individual or groups of colonies from any pool identified as being positive.

In the second stage of cloning, COS cells were co-transfected with the sublibrary pools and the 3/4FT gene by the same procedure used in the initial steps of screening. Forty-eight hours post-transfection, the transfected cells were screened using the fluorescent CHO:P-selectin assay of Example 4(B). Positive pools were further subdivided, as described above, until finally individual colonies were

screened and positive clones identified. Using this method, a single positive clone, pMT21:PL85, was found to encode the P-selectin ligand protein. The DNA sequence of the P-selectin ligand contained in pMT21:PL85 is set forth in SEQ ID NO:1, and the binding characteristics of the P-selectin ligand protein encoded by pMT21:PL85 are set forth in Example 4(C) below.

EXAMPLE 2

CLONING THE α 1,3/1,4 FUCOSYLTRANSFERASE GENE

The α 1,3/1,4 fucosyltransferase gene (3/4FT) was cloned from total human genomic DNA (Clontech Laboratories) by means of PCR. The sense oligonucleotide primer contained an XbaI site and the 5' terminus of the gene (5'-TAGCATACGCTCTAGAGCATGGATCCCCTGGGTGCAGCCAAGC-3', SEQ ID NO:9), and the antisense oligonucleotide primer contained an EcoRI site and the 3' terminus of the gene (5'-CCGGAATTCTCAGGTGAACCAAGCCGC-3', SEQ ID NO:10). The PCR product was sequentially digested with XbaI and EcoRI and purified by standard gel purification methods. This gene was then ligated with vector pMT3Sv2ADA (R. Kaufman, Methods in Enzymology, supra) that had also been sequentially digested with XbaI and EcoRI and purified by standard gel purification methods. Competent HB101 cells (Biorad) were transformed with this ligation product and then plated on agar plates in the presence of ampicillin. Nitrocellulose filter lifts of ampicillin-resistant transformants were probed with a radiolabelled oligonucleotide (5'-AAGTATCTGTCCAGGGCTTCCAGGT-3', SEQ ID NO:11) complementary to the nucleotide region 506-530 in the middle of the gene (J. Sambrook et al., supra).

Plasmid DNA minipreps were prepared from twelve positive clones. The purified DNA was then digested with EcoRI and XbaI to identify the correct clone with the proper size insert. This clone (pEA.3/4FT) was then grown up large scale and the DNA isolated by CsCl density

gradient banding (J. Sambrook et al., *supra*). DNA sequencing confirmed the identity of the 3/4FT gene. The functionality of the gene was assessed in a cell-cell binding assay as follows. COS-1 monkey cells [(clone M6; M. Horwitz et al., *Mol. Appl. Genet.*, 2:147-149, (1983)] were transfected with 3/4FT using DEAE dextran followed by DMSO shock treatment and chloroquine incubation [L. Sompeyrac and K. Dana, *Proc. Natl. Acad. Sci.*, 78:7575-7578 (1981); M. Lopata et al., *Nucleic Acids Res.*, 12:5707-5717, (1984); H. Luthman and G. Magnuson, *Nucleic Acids Res.*, 11:1295-1308, (1983)]. The transfected COS cells were suspended and quantitated for binding to a CHO line expressing E-selectin [G. Larsen et al., *J. Biol. Chem.* 267:11104-11110, (1992)]. This assay confirmed that the COS cells transfected with 3/4FT can express the sialylated Lewis^x epitope on the cell surface.

EXAMPLE 3

20 EXPRESSION OF THE P-SELECTIN LIGAND PROTEIN

A. Expression of the P-selectin Ligand in LEC11 cells

Functional P-selectin ligand was expressed in the SLe^x-positive Chinese hamster ovary (CHO) cell line LEC11 (Campbell, C. and Stanley, *P.Cell* 35:303-309 (1983) as follows: approximately 8 µg of plasmid containing the P-selectin ligand gene (pMT21:PL85, Example 1) was transfected into LEC11 cells. At 68 hours post-transfection, the cells were treated with 2.5 mM sodium butyrate for 4 hours. The cells were observed to induce P-selectin adhesion, as determined using the 6-CFD labeled CHO:P-selectin cell binding assay (described in Example 4, section B). In contrast, neither LEC11 cells alone nor LEC11 cells transfected with a control plasmid induced P-selectin adhesion.

B. Expression of Soluble P-Selectin Ligand in COS cells

COS cells were transfected with 8 μ g pED.sPSL.T7 (see Example 5C) and 4 μ g pEA.3/4 FT plasmid of Example 2, 8 μ g pED.sPSL.T7 alone, or 8 μ g plasmid vector (pMT21) and 4 μ g pEA.3/4 FT gene. Forty-five hr post-transfection, the cells were rinsed twice in PBS and incubated overnight at 37°C in serum-free DMEM minus phenol red (JRH Biosciences) supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. Phenylmethylsulfonyl fluoride, aprotinin and NaN₃ were added to final concentrations of 1mM, 2 μ g/ml and 0.02%, respectively, and the conditioned medium was centrifuged to remove all debris.

For immunoprecipitation experiments, the labeled soluble P-selectin ligand protein was produced by co-transfecting COS cells with pED.sPSL.T7 and pEA.3/4 FT. At forty-five hr post-transfection, the COS cells were labeled with 250 μ Ci/ml ³⁵S methionine (NEN) for 5 hours and the medium was collected. Expression of sPSL.T7 protein was confirmed by immunoprecipitation with anti-T7 antibodies.

C. Expression of PACE-cleaved P-selectin ligand in COS Cells

COS cells were co-transfected with the pED.sPSL.T7 plasmid of Example 5(C), the pEA.3/4FT cDNA of Example 2, and a plasmid containing the PACE cDNA as set forth in SEQ ID NO:5. A parallel control co-transfection was done using only the pED.sPSL.T7 plasmid and the pEA.3/4FT plasmid. After 45 hours, conditioned medium from these transfected COS cells was coated onto plastic dishes and binding to CHO:P-selectin cells (Example 4) was determined. An approximately two-fold increase in bound CHO:P-selectin cells was observed for dishes coated with medium containing the P-selectin ligand co-expressed with PACE, as compared with medium containing P-selectin ligand which had not been co-expressed with PACE. Amino acid sequencing of the N-terminus of purified sPSL.T7 protein from the PACE co-transfection showed that all of the ligand had been cleaved

at the PACE consensus site (amino acids 38-41 of SEQ ID NO:1). Radiolabelling of co-transfected COS cells with ³⁵S-methionine and subsequent SDS-polyacrylamide gel electrophoresis and autoradiography showed that comparable quantities of the P-selectin ligand had been secreted in both co-transfections.

D. Expression of the P-selectin Ligand Protein in CHO Cells

A full-length form (amino acids 1-402) of the P-selectin ligand protein was expressed in the CHO(DUKX) cell line (Urlaub & Chasin, Proc. Natl. Acad. Sci. USA 77, 4216-4220 (1980)) as follows: approximately 25 µg of the pMT21:PL85 plasmid and approximately 8 µg of the pED.3/4FT (produced by restriction of pEA.3/4FT with EcoRI and XbaI and insertion of the resulting fragment into the pED plasmid) were co-transfected into CHO(DUKX) cells using the calcium phosphate method. Transfectants were selected for resistance to methotrexate. After two weeks, individual colonies were screened for SLe^x expression by using a conjugate of an anti SLe^x antibody (CSLEX-1, U.S. 4,752,569) and sheep red blood cells (sRBC) prepared by the chromic chloride method (Goding, J. W., J. Immunol. Methods 10:61-66 (1976) as follows: sRBC were washed with 0.15M NaCl until the wash became clear and then a 50% suspension of sRBC was prepared in 0.15M NaCl. One ml of 0.01% chromic chloride solution was added dropwise while vortexing to 0.2 ml of a sRBC suspension containing 50 µg of CSLEX-1. After incubating at 37°C for 30 minutes, 10 ml of phosphate buffered saline (PBS) solution was added to the reaction. The conjugate was washed once before resuspending into 10 ml of PBS. The plates containing transfectants were washed with PBS and then 3 ml of PBS and one ml of the sRBC/CSLEX-1 conjugate was added to each plate. Positive colonies were red on a transilluminator and were picked into alpha medium with 10% fetal bovine serum. After two weeks, colonies were subjected to stepwise amplification using

methotrexate at concentrations of 2, 10, 25, 100, 250 nM. The stable cell line obtained was designated CD-PSGL-1 (R3.4). Expression of the P-selectin ligand protein was confirmed by immunoprecipitation studies using the polyclonal anti-P-selectin ligand protein antibody of Example 7(A). The functionality of the P-selectin ligand protein produced by the CD-PSGL-1 (R3.4) cell line was tested by assaying the transfectants for binding to LEC-γ1 as in Example 4(A).

10 The sPSL.T7 protein was expressed in a stable CHO-PACE line which was already expressing the cDNA encoding PACE as set forth in SEQ ID NO:5 under adenosine deaminase selection (Kaufman, et al., PNAS (USA) 83:3136-3140 (1986)). The psPSL.T7 (25 μg) and pED.3/4FT (8 μg) 15 plasmids were cotransfected into CHO-PACE cells using the calcium phosphate method. Transfectants were selected for resistance to methotrexate, and individual colonies which bound to the sRBC/CSLEX-1 conjugate were picked. After two weeks in culture, the colonies were subjected to stepwise 20 amplification as described above. The stable cell line obtained was designated CP/PSL-T7 (R4.1). Expression of sPSL.T7 protein was confirmed by standard immunoprecipitation methods using either a T7 specific monoclonal antibody or the LEC-γ1 chimera of Example 4(A). 25 In a similar fashion, a stable cell line expressing the mature full length form (amino acids 42-402) of the P-selectin ligand protein was obtained by co-transfection of pMT21:PL85 and pED.3/4FT into the CHO-PACE line.

Stable cell lines expressing the sPSL.Q protein of Example 5(B) and the sPSL.Fc protein of Example 5(D) were 30 constructed as follows: plasmids pED.sPSL.Q (25 μg) or pED.sPSL.Fc (25 μg) were cotransfected with approximately 25 μg of the pED.3/4FT plasmid described above and approximately 20 μg of a plasmid containing the PACE cDNA 35 as set forth in SEQ ID NO:5) as well as the neomycin resistance gene into CHO(DUKX) cells using the calcium phosphate method. Transfectants were selected for

resistance to methotrexate and the G418 antibiotic. Approximately two weeks later, individual colonies were screened for SLe^x expression using sRBC/CSLEX-1 conjugate binding. The positive colonies were picked in G418 medium at 1 mg/ml concentration. After 2-3 weeks in culture, cells were amplified with methotrexate in a stepwise selection. The stable cell lines obtained were designated CD-sPSL.Q (R8.2) and CD-sPSL.Fc (R8.1), respectively. The expression of sPSL.Q and sPSL.Fc protein was confirmed by standard immunoprecipitation method using the anti P-selectin ligand protein polyclonal antibody of Example 7(A).

EXAMPLE 4

ASSAYS OF P-SELECTIN-MEDIATED INTERCELLULAR ADHESION

A. LEC-γ1 Binding Assay

A DNA encoding a chimeric form of P-selectin conjugated to the Fc portion of a human IgGγ1 (LEC-γ1) was constructed using known methods (Aruffo et al. Cell 67, 35-44 (1991)), and stably transfected into dhfr^r CHO cells (CHO DUKX) for high level production of the chimeric LEC-γ1 protein, which was purified for use in the binding assay set forth below.

Petri dishes were coated first with a polyclonal anti-human IgGγ1 Fc antibody and then with LEC-γ1. This method orients the LEC-γ1 construct such that the P-selectin portion of the chimeric molecule is presented on the surface of the plates. Adhesion of HL60 cells to the oriented LEC-γ1 was quantitated in the presence and absence of calcium. HL60 adhesion was shown to be calcium dependent, confirming that the chimeric molecule had retained functional binding of P-selectin to its ligand on HL60 cells. The binding of HL60 cells to oriented LEC-γ1 was also shown to be blocked by a neutralizing monoclonal

antibody to P-selectin, demonstrating the specificity of P-selectin binding.

B. Fluorescent CHO-P-selectin Binding Assay

5 The assay employed a fluorescently labeled CHO:P-selectin cell line (Larsen et al., J. Biol. Chem. 267, 11104-11110 (1992)) that can bind to and form clusters on the surface of COS cells that are co-transfected with the P-selectin ligand gene and the 3/4 FT gene. The CHO:P-selectin cells were suspended at 1.5×10^6 cells/ml in 1% fetal bovine serum in DME medium and labeled by adding 6-carboxyfluorescein diacetate (6-CFD) to a final concentration of 100 ug/ml. After incubation at 37°C for 15 minutes, the cells were washed in medium and resuspended at 1×10^5 cells/ml. Five ml of the labeled cells were added to each washed COS transfectant-containing plate to be assayed and incubated at room temperature for 10 minutes. Nonadherent cells were removed by four washes with medium. The plates were then scanned by fluorescence microscopy for rosettes of adherent CHO:P-selectin cells.

C. Quantitative adhesion assay using radioactively labeled CHO:P-selectin cells

25 COS cells were co-transfected with the pMT21:PL85 plasmid of Example 1 and the pEA.3/4FT plasmid of Example 2 by the same procedure used in the initial stages of screening. As controls, COS cells were transfected with pMT21:PL85 alone, or with pEA.3/4FT alone, or with a similar plasmid containing no insert ("mock"). 24 hours post-transfection, the transfected cells were trypsinized and distributed into Costar 6-well tissue culture plates. CHO:P-selectin cells were labeled for 16 hours with ^3H -thymidine using known methods and preincubated at 0.5×10^6 cells/ml for 30 minutes at 4°C in α medium containing 1% BSA (control); α medium containing 1% BSA, 5 mM EDTA and 5 mM EGTA; α medium containing 1% BSA and 10 $\mu\text{g/ml}$ of a neutralizing anti P-selectin monoclonal antibody; and α medium containing 1% BSA and a non-neutralizing anti-P-

selectin monoclonal antibody. The preincubated cells were then added to the wells containing the transfected COS cells. After a 10 minute incubation, unbound cells were removed by 4 changes of medium. The bound CHO:P-selectin
5 cells were released by trypsinization and quantified by scintillation counting.

COS cells co-transfected with P-selectin ligand and the 3/4FT induced approximately 5.4-fold more binding of CHO:P-selectin cells relative to COS mock cells; assay in
10 the presence of EGTA and EDTA reduced binding to the level of the mock transfected COS cells. Likewise, incubation with neutralizing anti-P-selectin antibody also eliminated specific binding, whereas non-neutralizing antibody had no effect. In contrast, the binding of CHO:P-selectin to COS
15 cells transfected with P-selectin ligand alone was not statistically different than binding to the mock-transfected COS in both the presence or absence of EDTA and EGTA, or anti-P-selectin antibodies. The binding of CHO:P-selectin cells to COS cells transfected with 3/4 FT alone
20 was approximately 2-fold greater than to the mock-transfected COS, but was unaffected by the presence or absence of EDTA and EGTA.

EXAMPLE 5

25 CONSTRUCTION OF SOLUBLE P-SELECTIN LIGANDS

The EcoRI adaptors used to generate the cDNA library from HL60 cells in Example I contain an XbaI restriction site (TCTAGA) just 5' of the beginning of SEQ ID NO:1 as it is located in the pMT21:PL85 plasmid. In order to generate
30 soluble forms of the PSL, the pMT21:PL85 plasmid was restricted with XbaI and with HincII (which cleaves after nucleotide 944 of SEQ ID NO:1). The approximately 950 bp fragment thus generated, containing all of the encoded extracellular segment of the ligand up to and including the
35 codon for valine 295, was isolated and used to generate DNAs encoding soluble forms of the P-selectin ligand protein as set forth in sections A through D below.

A. Construction of psPSL.QC

The fragment was purified and ligated into mammalian expression vector pED between the XbaI and EcoRI sites, along with double stranded synthetic oligonucleotide DNA that recreated the codons from Asn 296 to Cys 310 and introduced a novel stop codon immediately following Cys 310. The sequence of the oligos is as follows:
5'-AACTACCCAGTGGGAGCACCAGACCACATCTCTGTGAAGCAGTGCTAG (SEQ ID NO:12)

5'-AATTCTAGCACTGCTTCACAGAGATGTGGTCTGGTGCTCCCACTGGGTAGTT (SEQ ID NO:13)

The resulting plasmid was designated pED.sPSL.QC, and the protein expressed from the plasmid was designated sPSL.QC.

B. Construction of psPSL.Q

The fragment was purified and ligated into the pED plasmid (Kaufman et al., 1991) between the XbaI and EcoRI sites, along with the double stranded synthetic oligonucleotide DNA that recreated the codons from Asn 296 to Gln 309 and introduced a novel stop codon immediately following Gln 309. The sequence of the oligos is as follows:

5'-AACTACCCAGTGGGAGCACCAGACCACATCTCTGTGAAGCAGTAG (SEQ ID NO:14)

5'-AATTCTACTGCTTCACAGAGATGTGGTCTGGTGCTCCCACTGGGTAGTT (SEQ ID NO:15)

The resulting plasmid was designated pED.sPSL.Q, and the protein expressed from the plasmid was designated sPSL.Q.

C. Construction of psPSL.T7

Oligonucleotides encoding 14 amino acids including an epitope derived from the phage T7 major capsid protein were synthesized, creating a C-terminal fusion of the epitope "tag" with an additional 32 amino acids derived from the vector sequence. Two oligonucleotides having the sequences
5'-CTAGACCCGGGATGGCATCCATGACAGGAGGACAACAAATGGTAGGCCGTAG (SEQ ID NO: 16) and

5'-AATTCTACGGCCTACCCATTTGTTGTCCTCCTGTCATGGATGCCATCCCGGGT
(SEQ ID NO:17)

were duplexed and ligated with the large XbaI-EcoRI
fragment of mammalian expression plasmid pED. The
5 resulting plasmid, pED.T7 was restricted with XbaI and SmaI
and ligated to the 950 bp XbaI-HincII fragment described
above, resulting in plasmid pED.sPSL.T7.
The protein resulting from expression of pED.sPSL.T7 was
designated sPSL.T7.

10 **D. Construction of Soluble P-selectin Ligand--IgGFc
Chimera**

The plasmid DNA encoding a soluble, extracellular form
of the P-selectin ligand protein fused to the Fc portion of
human immunoglobulin IgG1 was constructed as follows: the
15 mammalian expression vector pED.Fc contains sequences
encoding the Fc region of a human IgG1 with a novel linker
sequence enabling the fusion of coding sequences amino
terminal to the hinge region via a unique XbaI restriction
site. A three fragment ligation was performed: pED.Fc was
20 restricted with XbaI and gel purified in linear form. The
950 bp fragment from pMT21:PL85 described above comprised
the second fragment. The third fragment consisted of
annealed synthetic oligonucleotide DNAs having the
following sequence:

25 5' - CTGCGGCCGCGAGT (SEQ ID NO:18)

5' - CTAGACTGCGGCCGCGAG (SEQ ID NO:19)

The ligation products were grown as plasmid DNAs and
individual clones having the correct configuration were
identified by DNA sequencing. The plasmid was designated
30 pED.PSL.Fc. The DNA coding region of the resulting soluble
P-selectin ligand /Fc fusion protein is shown in SEQ ID
NO:6.

EXAMPLE 6

CHARACTERIZATION OF EXPRESSED P-SELECTIN LIGANDS

A. Binding Characterization of Full-Length P-selectin Ligand Protein Expressed on COS Cells

Co-transfection of COS cells with the pEA.3/4FT plasmid of Example 2 and the pMT21:PL85 plasmid of Example 1 yields COS cells which specifically bind to CHO:P-selectin cells. This binding is observed only upon co-transfection of pEA.3/4FT and pMT21:PL85; use of either plasmid alone generates COS cells which do not bind to CHO:P-selectin cells. No binding is observed between the parental CHO(DUKX) cell line which does not express P-selectin and COS cells co-transfected with pEA.3/4FT and pMT21:PL85. The binding between the co-transfected COS cells and CHO:P-selectin cells is sensitive to chelators of divalent ions such as EDTA and EGTA, consistent with the Ca^{++} dependency of P-selectin mediated cellular adhesion. A neutralizing anti-P-selectin monoclonal antibody blocked the binding between the CHO:P-selectin cells and the COS cells which had been co-transfected with pEA.3/4FT and pMT21:PL85, while a non-neutralizing anti-P-selectin monoclonal antibody had no effect on the binding. The antibody results indicate that the functional domain(s) of P-selectin are required for binding to P-selectin ligand protein expressed on the surface of COS cells.

B. Electrophoretic Characterization of Full-Length P-selectin Ligand Expressed in COS Cells

Detergent extracts of co-transfected COS cells were prepared as follows: 45 hours post co-transfection, approximately 1.5×10^7 cells were suspended in 5 ml of lysis buffer (10mM Piperazine-N,N'-bis[2-ethanesulfonic acid] (PIPES) pH 7.5, 100 mM KCl, 3 mM $MgCl_2$, 1 mM benzamidine, 0.5 μ g/ml leupeptin, 0.75 μ g/ml pepstatin, 1 mM ethylmaleimide, and 1 μ g/ml aprotinin) and lysed by sonication. Cellular debris was removed by low speed centrifugation (500 x g. 10 minutes), and a membrane fraction collected by ultracentrifugation (100,000 x g, 60

min). The high speed membrane pellet was resuspended in an extraction buffer (10 mM 3-[N-Morpholino]propanesulfonic acid) (MOPS) pH 7.5, 0.1 M NaCl, 0.02% NaN₃, 1% Thesit® (Sigma), 1 mM benzamidine, 0.5 µg/ml leupeptin, 0.75 µg/ml pepstatin, 1 mM ethylmaleimide, and 1 µg/ml aprotinin). Samples were then subjected to SDS polyacrylamide gel electrophoresis and transfer to nitrocellulose blots as follows: an aliquot of the detergent extract was suspended in 1% SDS loading buffer and heated for 5 minutes at 100°C before loading onto an 8-16% polyacrylamide gel (reduced) or a 6% gel (non-reduced) and electrophoresed in the Laemmli buffer system. Blots were prepared using Immobilon-P® transfer membranes. The blots were immersed in 10 mM MOPS pH 7.5, 0.1 M NaCl, 0.02% NaN₃, 1 mM MgCl₂, 1 mM CaCl₂, and 10% non-fat milk overnight at 4°C. Blots were rinsed once in the above buffer, minus the milk, and incubated in blotting buffer (10 mM MOPS pH 7.5, 0.1M NaCl, 1% bovine serum albumin, 0.05% Thesit, 1 mM MgCl₂, 1 mM CaCl₂) for 30 minutes at room temperature.

The blots were then probed for the P-selectin ligand as follows: 50 ng of a P-selectin/Fc chimera was pre-incubated with 3 µCi of ¹²⁵I-Protein A in blotting buffer for 30 minutes at room temperature. Additional excipients (e.g., EDTA, EGTA, monoclonal antibodies) could be added to the pre-incubation mixture at this point to evaluate their effects on binding of the chimera to the P-selectin ligand. The pre-incubated mixture was then incubated with the blots (prepared as above) for 60 minutes at room temperature, and the blots were subsequently washed four times with the same blotting buffer (without bovine serum albumin), air dried, and autoradiographed at -70°C.

Under non-reducing conditions, two bands were observed with this technique for membrane extracts prepared from co-transfected COS cells. The major band migrated with an estimated molecular weight of approximately 220 kD, whereas the minor band migrated with a molecular weight of approximately 110 kD. Under reducing conditions, only a

single band was observed with a molecular weight of approximately 110 kD, indicating that under non-reducing conditions, the P-selectin ligand exists as a homodimer. The approximate molecular weight of the reduced monomer is greater than that predicted from the deduced amino acid sequence of the cDNA clone (45 kD), indicating that the expressed protein undergoes extensive post-translational modification (see Example 6(C)). The specificity of the P-selectin/Fc chimera was confirmed by the observation that a nonspecific IgG₁ probe yielded no bands on the blots. Additionally, the binding of the P-selectin/Fc chimera to the blots was abolished by EDTA, EGTA, and a neutralizing anti-P-selectin monoclonal antibody. Specific bands on the blots were observed only from membrane extracts of COS cells co-transfected with the pEA.3/4FT and pMT21:PL85 plasmids. Membrane extracts from control transfections (pEA.3/4FT or pMT21:PL85 alone) failed to yield observable bands on blots.

20 C. Glycosylation of P-selectin Ligand Protein

The presence of covalently attached carbohydrate on recombinant P-selectin ligand and its role in binding to P-selectin was determined as follows: COS cells were co-transfected with pED.sPSL.T7 of Example 5(C) and the pEA.3/4FT plasmid of Example 2. After 48 hours, the cells were pulsed with ³⁵S-methionine. 200 μ l of ³⁵S methionine-labeled sPSL.T7 conditioned medium was incubated with 5 μ g LEC- γ 1 in the presence of 2 mM CaCl₂ and 1 mg/ml bovine serum albumin (BSA). After rotating for 2 hours at 4°C, Protein A-Sepharose beads (Pharmacia) were added for 1 hour at 4°C, pelleted by centrifugation and washed twice in Tris buffered saline (20 mM Tris-HCl, 150 mM NaCl pH 7.5, hereinafter TBS) containing 2 mM CaCl₂ and 1 mg/ml BSA. The pellets were then resuspended and treated with neuraminidase (*Streptococcus pneumoniae*), O-glycanase, and N-glycanase (all from Genzyme) as follows. All glycosidase digestions were done at 37°C overnight. For neuraminidase

digestion, the pellet was resuspended in 50 μ l 2-(N-morpholino)-ethanesulfonic acid (MES) buffer, pH 6.5 (Calbiochem) and 0.1% SDS, heated at 95°C for 5 minutes, then pelleted. The supernatant was modified to contain
5 1.4% n-Octyl B-D-glucopyranoside (OGP), 10mM calcium acetate, 20 mM sodium cacodylate and 2.5 mM PMSF, final pH 7.0 Eight μ l neuraminidase was added for a final concentration of 1 unit/ml. For neuraminidase/O-glycanase digestion, the sample was prepared as above and along with
10 the neuraminidase, the O-glycanase was also added to a final concentration of 0.1 unit/ml. For N-glycanase digestion, the pellet was resuspended in 54 μ l MES buffer and 1% SDS, heated at 95°C for 5 minutes, then pelleted. The supernatant was modified to contain 0.2 M sodium
15 phosphate, 3.5% OGP, and 2.5 mM PMSF, final pH 8.5. N-glycanase was added for a final concentration of 12 units/ml and incubated as above.

The effect of glycosidase treatment on SPSL.T7 was assessed in two ways. For this, each digested protein
20 sample was divided into two equal fractions. One fraction was precipitated with the P-selectin polyclonal antibody of Example 7(A), to show the effect of digestion on the electrophoretic mobility. The other fraction was precipitated with the LEC- γ 1 chimera of Example 4(A), to
25 assess the remaining P-selectin ligand binding activity after digestion. The immunoprecipitated samples were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions and autoradiography.

In the absence of glycosidase treatment,
30 autoradiography revealed comparable bands (with molecular weights of 110 kD) for each precipitation. When the P-selectin ligand protein was treated with neuraminidase, anti-P-selectin ligand polyclonal antibody precipitation revealed a slight decrease in mobility, consistent with
35 removal of sialic acid residues. The amount of P-selectin ligand protein precipitated by LEC- γ 1 was significantly reduced after neuraminidase treatment, consistent with the

role of sialic acid residues in the P-selectin/P-selectin ligand interaction. When the P-selectin ligand protein was treated with both neuraminidase and O-glycanase, a substantial increase in electrophoretic mobility was observed after precipitation with the anti-P-selectin ligand polyclonal antibody, indicating that a number of O-linked oligosaccharide chains had been removed. However, removal of O-linked oligosaccharides from the P-selectin ligand protein may not have been complete, since the electrophoretic mobility did not correspond to a protein with a molecular weight of 38 kD, as would be predicted from the amino acid sequence set forth in SEQ ID NO:1. The neuraminidase/O-glycanase digested P-selectin ligand protein bound to LEC- γ 1 very poorly, further indicating the role of oligosaccharides in the P-selectin/P-selectin ligand interaction. Treatment of the purified P-selectin ligand with N-glycanase resulted in a slight increase in electrophoretic mobility, demonstrating that some of the consensus sites for N-linked glycosylation are occupied. The amount of P-selectin ligand protein precipitated by LEC- γ 1 was slightly reduced, indicating that N-linked glycosylation also contributes to the P-selectin/P-selectin ligand interaction, though not as dramatically as sialylation and O-linked glycosylation.

EXAMPLE 7

POLYCLONAL ANTIBODIES SPECIFIC FOR P-SELECTIN LIGANDS

A.	<u>Polyclonal</u>	<u>Rabbit</u>	<u>anti-P-selectin</u>	<u>Ligand</u>
	<u>Protein/Maltose</u>	<u>Binding Protein</u>	<u>Fusion Protein</u>	<u>Protein</u>

The anti-P-selectin ligand polyclonal antibody was generated by immunizing rabbits with a fusion protein generated in *E. coli*. The fusion protein consisted of the amino terminal one-third of the P-selectin ligand (amino acids 1 to 110 of SEQ ID NO:1) fused in frame to the maltose binding protein (Maina, C. V. et al., Gene 74, 365-373 (1988); Riggs, P., in Current Protocols in Molecular Biology, F. M. Ausubel et al., Eds., Greene

Associates/Wiley Interscience (New York, 1990) chapter 16.6). Under conditions employed herein, the fusion protein antibody recognizes the P-selectin ligand protein.

5 **B. Polyclonal Rabbit Anti-sPSL.T7 Protein**

 A soluble form of the invention (sPSL.T7; see example 5(C)) was purified to apparent homogeneity according to the following scheme: COS cells were transfected with three plasmids, one encoding each of the following: sPSL.T7
10 (Example 5(C)), 3/4FT (Example 2), and a soluble form of PACE (as set forth in SEQ ID NO:5). After 72 hours, the conditioned medium was collected and recombinant sPSL.T7 was purified as follows.

 Conditioned medium was diluted two fold with 50 mM
15 MOPS, 150 mM NaCl, 0.5 mM CaCl₂ and 0.5 mM MnCl₂, pH 7.2, and applied to a column of lentil lectin-Sepharose 4B equilibrated in the same buffer. After loading, the column was washed with the same buffer until the optical absorbance at 280 nm dropped to a stable baseline. The
20 column was then eluted with the same buffer which had been adjusted to 0.5 M α -methyl-mannoside and 0.3 M NaCl. Recombinant sPSL.T7 was collected over 5-15 column volumes of this elution buffer. The lentil lectin eluate was then subjected to a 0-70% ammonium sulfate precipitation by
25 adding 472g of ammonium sulfate per liter of column eluate at 4°C. After stirring for 30 minutes, the precipitate was resuspended in a minimal volume of TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) and applied to a TSK G4000SW_{XL} gel filtration column equilibrated in TBS. The flow rate on
30 the column was 0.5 ml/min and a guard column was employed. In aliquots of < 250 μ l, the resuspended ammonium sulfate pellet was injected on the column and fractions analyzed by SDS-PAGE with Western analysis. Fractions containing sPSL.T7 were pooled and then used for immunizing rabbits.

35 Antibodies to sPSL.T7 were generated in the standard fashion by antigen priming and subsequent boosting over a 3 month period. Specifically, primary immunization was

performed by mixing 50 μ g of sPSL.T7 (denatured by mixing in 0.1% SDS and heating for 10 minutes at 100°C) with complete Freund's adjuvant and injected at five sites subcutaneously. The second (and all subsequent) boosts
5 were performed by mixing 25 μ g of sPSL.T7 (denatured by mixing in 0.1% SDS and heating for 10 minutes at 100°C) [12.5 μ g for the third and subsequent boosts] with incomplete Freund's adjuvant and injecting at two sites subcutaneously (or later, intramuscularly) every two weeks.
10 Test bleeds were performed every two weeks to monitor antibody titer. When the antibody titer reached a suitable level, a larger scale bleed was performed and a total serum fraction prepared. This polyclonal antibody preparation was used to inhibit the specific binding of HL60 cells to
15 CHO:P-selectin cells in a manner similar to that described in Example 4.

This assay employed fluorescently-labeled HL60 cells (labelled with BCECFAM; 2',7'-bis-(2-carboxymethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester) binding to CHO
20 cells plated on the bottom of microtiter plates. The labelled HL60 cells were pre-incubated with either sera containing polyclonal antibody or with pre-immune sera for 30 minutes at 4°C. The cells were then washed and incubated with the CHO:P-selectin cells for 10 minutes. The plates
25 were then washed and the fluorescence read with a fluorescence microtiter plate reader. Using this assay, a 1:15 dilution of the anti-sPSL.T7 polyclonal serum resulted in essentially complete inhibition of HL60 cell binding to CHO:P-selectin. Demonstrable inhibition of HL60 binding to
30 CHO:P-selectin was still observed at antiserum dilutions of 1:150. Pre-immune serum had no effect on HL60 cell binding to CHO:P-selectin.

EXAMPLE 8

COTRANSFORMATION WITH CORE2

A. Isolation of the cDNA encoding Core2 GlcNAc Transferase

5 The cDNA encoding core2 GlcNAc transferase was isolated by standard molecular biology techniques. Two oligos were designed at the 5' and 3' end (including translational initiation and termination codon, respectively) based on the published human core2 sequence
10 (Bierhuizen, M.F.A., Fukuda, M., Proc. Natl. Acad. Sci. 89, 9326-9330 (1992)). The pools of an HL60 cDNA library (Sako, D., Cell 75, 1179-1186 (1993)) were used as template to amplify the core2 coding sequence by a standard PCR protocol. The PCR amplified fragment was purified and
15 subcloned into pED vector. To isolate cDNA, the pools which gave a positive signal in the PCR reaction were transformed into *E. coli* and plated. Transformants were transferred onto nitrocellulose filters and hybridized with a ³²P radiolabelled PCR fragment according to standard
20 protocols. Positive clones were picked and purified by replating. The sequence of the cDNA and PCR clone was confirmed by dideoxy sequencing.

B. Generation of Stable PSGL-1 Chinese Hamster Ovary Cell Lines Expressing Core2 Enzyme

25 A cell line made in accordance with the methods of Example 3 expressing full-length P-selectin ligand protein and 3/4 fucosyltransferase was co-transfected with core2 cDNA and a neomycin resistance gene (pMT4Neo) by standard calcium phosphate methods. After about two weeks, stable
30 G418-resistant transfectants were picked either as single isolates or in a pool. These transfectants were grown in 1 mg/ml G418 complete DMEM media and analyzed for core2 enzyme activity (Higgins, E.A., et al., J. Biol. Chem. 266, 6280-6290 (1991)). Positive clones or pools found positive
35 for core2 activity were analyzed for P-selectin ligand binding to P-selectin by various methods. In a similar fashion, cell lines expressing either P-selectin ligand

protein or soluble P-selectin ligand protein with both the 3/4 fucosyltransferase and PACE enzymes (see Example 3) were used to isolate stable cotransfectants of core2 as described above.

5 C. Effects of Core2 on P-selectin Binding Activity

The effects of core2 on P-selectin binding activity was evaluated by three different methods:

1. Binding of mPSGL-1 transfectants to immobilized soluble P-selectin or P-selectin/IgG chimera.

10 48- well plates were coated with 1 ug/ml anti human Fc antibody in 50 mM Tris pH 9.5 at 4° C for five to six hours. After washing twice with HBSS buffer, P-selectin/IgG chimera (0.1-1 ug/ml conc., Example 5) was plated in HBSS buffer overnight at 4° C. The plates were blocked with BSA
15 (3 mg/ml) at 4° C for three to four hours. In the case of soluble P-selectin ligand protein, the protein was coated directly onto plates in the same buffer. The ³H labelled CHO cells were lifted with 2 mM EGTA, washed three times with PBS, and resuspended to a final density of 10⁶
20 cells/ml. A 300 ul aliquot of this suspension was added to each well (300,000 cell/well). After incubating for 12 minutes at room temperature, wells were washed four times with serum free DMEM to remove unbound cells. Bound cells
25 were lifted with 5 mM EGTA and counted in scintillation counter. U937 cells, used as a positive control for native P-selectin ligand protein binding, were pretreated with gamma globulin (5 mg/ml) to block endogenous Fc receptor before binding to P-selectin IgG chimera. Comparative binding data are shown in Fig. 1.

30 2. Immunoprecipitation of PSGL-1 with P-selectin/IgG Chimera.

Recombinant full-length or soluble P-selectin ligand protein prepared from transformants, with and without additional core2, was labelled with ³⁵S-methionine and
35 subsequently immunoprecipitated with either the anti P-selectin ligand protein polyclonal antibody or P-selectin ligand/IgG chimera as described previously in Examples 7

and 5; Sako, D., Cell 175, 1179-1186 (1993). Data are depicted in Fig. 2.

3. Flow Cytometry.

Stable murine P-selectin ligand protein transfectants (with and without core2) were analyzed by standard FACS techniques using either P-selectin/IgG chimera (LecY1) (Example 5) or anti P-selectin ligand protein monoclonal antibody (MAb 275, raised against a peptide having the sequence from amino acid 42 to amino acid 56 of SEQ ID NO:2). Both reagents were preconjugated to FITC labelled Protein A. Cells were analyzed by FACS after incubating with this conjugate for 30 minutes at 4° C in the presence of 2 mM CaCl₂. Data are depicted in Fig. 3.

EXAMPLE 9

E-SELECTIN BINDING OF P-SELECTIN BINDING PROTEIN

E-Selectin/IgG chimera was made as described in Example 5 for the P-selectin IgG chimera using an E-selectin encoding DNA including amino acids -21 to 536 of the sequence reported in Bevilacqua et al., Science, 243:1160 (1989).

U937 cells (approximately 6.5×10^7) were recovered from tissue culture plates and divided equally into two 50 mL cultures (final concentration of 1.3×10^6 cells/mL) containing fresh complete RPMI medium and 50 μ Ci/ml of ³H-glucosamine hydrochloride (labels the protein-linked carbohydrate of glycoproteins [Varki, FASEB 5:226-235 (1991)]). After 48 hours incubation, the cells from both cultures were recovered by centrifugation and washed three times with PBS. The pelleted cells were suspended in 2.5 mL each of a lysis buffer containing 1% Triton X-100 and disrupted by probe sonication for two minutes. The detergent lysates were placed on ice for three hours and then resonicated for an additional two minutes. The lysates were centrifuged at 16,000 rpm for five minutes, the supernatants were recovered and each adjusted to 12 mL with lysis buffer containing no detergent. To one of the

two diluted cell lysates was added 100 uL of protein A
sepharose precoupled with P-selectin/IgG chimera (see
Example 5) and to the other was added 100 uL of protein A
sepharose precoupled with E-selectin/IgG chimera. Both
5 chimeric proteins were present at a density of
approximately 2 mg protein/mL of resin. Binding reactions
were allowed to proceed overnight at 4 degrees C with end-
over-end mixing. On occasion, purified membranes from U937
cells served as the starting material for the detergent
10 extraction of labeled proteins. In these cases, the
detergent extraction and affinity precipitation steps were
essentially identical to the above.

Following incubation, the two parallel reaction
mixtures were each centrifuged at 2,000 rpm and
15 supernatants were discarded. The resin pellets were washed
four times with buffer (10 mM MOPS, 100 mM NaCl, 1 mM CaCl₂,
1 mM MgCl₂, 0.02% NaN₃, pH 7.5 with Triton X-100 [0.25% for
the first and second washes, 0.1% for the third wash and
0.01% for the fourth wash]). A final 1 mL pre-elution wash
20 of each resin pellet using buffer containing 0.01% Triton
X-100 was conducted and these were retained for
quantitation of radioactive counts by liquid scintillation
counting (LSC). The resins were then eluted overnight at
4 degrees C with end-over-end mixing in 1 mL each of buffer
25 containing 0.01% Triton X-100 and 10 mM EDTA. The
supernatants were recovered by centrifugation and then
quantitated by LSC.

Autoradiography of the materials released from
the resins by EDTA was performed by electrophoresis of
30 samples (approximately 10,000 cpm samples concentrated by
Centricon-10 units where needed) on 10% cross-linked SDS-
PAGE gels, subsequent treatment of the gels with ENHANCE
(Dupont) as per the manufacturer's instructions followed by
drying for two hours on a commercially available gel dryer
35 (Bio-Rad). Exposure of the dried gels to X-ray film was
conducted for a minimum of three days at -80 degrees C.

Elution of immobilized E- or P-selectin, previously exposed to detergent extracts of U937 cells and exhaustively washed, with EDTA yielded liberated, ³H-glucosamine labeled proteins. The amount of radiolabel recovered from the EDTA eluates was at least 10-fold higher than the counts observed in the final, pre-EDTA washes. This observation suggests that both P- and E-selectin chimeras affinity captured ligand(s) from U937 whole cell lysates in an EDTA-dependent manner and that captured ligands were subsequently released upon treatment of the resins with EDTA.

The evaluation of the proteins released by EDTA from the two chimeras was performed by SDS-PAGE and autoradiography under reducing and non-reducing conditions (commercially available ¹⁴C-labeled molecular weight standards were employed). As shown by the autoradiograph depicted in Fig. 4, the released counts from the whole cell lysates treated with the P-selectin chimera (lanes 2 and 10) and the E-selectin chimera (lanes 4 and 8) correlated to a major species of 200 kD molecular weight, non-reduced (lanes 2 and 4), and 100 kD reduced (lanes 8 and 10). In different experiments depicted in Fig. 4, where purified membrane extracts were used as the starting material in place of whole cells, both the E-selectin chimera (lane 3, non-reduced and lane 9, reduced) and the P-selectin chimera (not shown) gave similar results. Other experiments have demonstrated that the major U937 glycoprotein which binds to P-selectin is immunoreactive with Rb3026, a polyclonal antibody raised against recombinant sPSGL1.T7. Therefore, P- and E-selectin specifically recognize a single major glycoprotein species with identical properties in each case.

Example 10**Production and Analysis of Deleted or Altered
Forms of Soluble P-Selectin Ligand Protein****A. Generation of DNA Constructs**

5 Truncated forms of the P-selectin ligand protein-IgG
chimeras were generated as follows. Plasmid pED.PSL.Fc was
restricted with PstI and NotI and the 6kb fragment
comprising the Fc portion and vector, pEDFc6kb, was gel
purified. Plasmid constructs pED.149.Fc, pED.47.Fc and
10 pED.19.Fc were created by standard PCR technique, using the
following pairs of oligonucleotide primers:

"Upstream" primer for all constructs:

5'-CCAGGTCCAACTGCAGGTCGACTCTAGAGGGCACTTCTTCTGGGCCCACG-3'
(SEQ ID NO:20)

15 "Downstream" primer for 148Fc:

5'-TATTATCTGTGCGGCCCGCCCTCCAGAACCCATGGCTGCTGGTTGCAGTGG-3'
(SEQ ID NO:21)

"Downstream" primer for 47Fc:

5'-TATTATCTGTGCGGCCCGCGCAGCAGGCTCCACAGTGGTAG-3' (SEQ ID
20 NO:22)

"Downstream" primer for 19Fc:

5'-TATTATCTGTGCGGCCCGCGGAGGCTCCGTTTCTGGCAG-3' (SEQ ID NO:23)

The template DNA for PCR reaction was pED.PSL.Fc. The PCR
conditions were 94°C, 1 min.; 42°C, 1 min.; 72°C, 3 min.;
25 25 cycles, using a Perkin-Elmer Thermocycler. After
completion of the last cycle, the reaction was treated with
Klenow enzyme at 25°C for 30 min., extracted with phenol
chloroform, sodium acetate added to 0.3M, and the PCR
product DNA was precipitated with 2.5 volumes of ethanol.
30 The DNA pellet was rinsed with 70% ethanol and residual
ethanol was evaporated. The resuspended DNA was digested
with PstI and NotI, gel purified and ligated with the
pEDFc6kb fragment described above. Correct constructs were
identified by restriction analysis and confirmed by DNA
35 sequencing.

Plasmid pED.ΔY148.Fc, pED.H24.Q70.148.Fc were created
by site directed mutagenesis (Maniatis et al., 1989,
Molecular Cloning: A Laboratory Manual, Cold Spring Harbor

Laboratories) using pED.148Fc as template and the following mutagenesis oligonucleotides:

for ΔY148: 5'-CGGAGACAGGCCACCGAATTCCTGCCAGAAACG-3' (SEQ ID NO:24)

5 for H24: 5'-CCTCCAGAAATGCTGAGGCACAGCACTGACACCACTCCTC-3' (SEQ ID NO:25)

for Q70: 5'-GAGCTGGCCAACATGGGGCAACTGTCCACGGATTCAGCAG-3' (SEQ ID NO:26)

Positive clones were identified by colony hybridization
10 (Maniatis et al, supra).

pED.FFFE.148.Fc was constructed by restricting pED.ΔY148.Fc with EcoRI and ligating the following duplexed oligonucleotides:

5'-AATTCGAGTTCCTAGATTTTG-3' (SEQ ID NO:27) and

15 5'-AATTCAAAATCTAGGAACTCG-3' (SEQ ID NO:28).

Constructs of the series pED.FYYD.19.Fc, pED.FFYD.19.Fc and pED.FFFD.19.Fc, were made by restricting pED.ΔY148.Fc with EcoRI and NotI and ligating the following duplexed oligonucleotides:

20 for pED.FYYD.19.Fc:

5'-AATTCGAGTACCTAGATTATGATTTCTGCCAGAACTGAGCCTCCGC-3' (SEQ ID NO:29) and

5'-GGCCGCGGAGGCTCAGTTTCTGGCAGGAAATCATAATCTAGGTACTCG-3' (SEQ ID NO:30);

25 for pED.FFYD.19.Fc:

5'-AATTCGAGTTCCTAGATTATGATTTCTGCCAGAACTGAGCCTCCGC-3' (SEQ ID NO:31) and

5'-GGCCGCGGAGGCTCAGTTTCTGGCAGGAAATCATAATCTAGGAACTCG-3' (SEQ ID NO:32);

30 for pED.FFFD.19.Fc:

5'-AATTCGAGTTCCTAGATTTCTGATTTCTGCCAGAACTGAGCCTCCGC-3' (SEQ ID NO:33) and

5'-GGCCGCGGAGGCTCAGTTTCTGGCAGGAAATCGAAATCTAGGAACTCG-3' (SEQ ID NO:34).

B. Plate Binding Assay for Analysis of Deleted or Altered Forms of Soluble P-Selectin Ligand Protein

The individual plasmid DNAs encoding the various mutated forms of soluble PSGL-1/Fc chimeras were co-transfected with pEA.3/4FT and PACE cDNA in COS cells as described in Example 3(c). 50mls of serum free medium, collected 40-64 hours post transfection from approximately 10^7 COS cells, was purified on a column of 0.25ml of protein A sepharose (Pharmacia) equilibrated with TBS supplemented with 2mM CaCl_2 . After washing with 20mls of TBS/ CaCl_2 , the bound material was eluted with 0.5mls of 0.1M acetic acid, 0.15M NaCl, 2mM CaCl_2 . The eluted material was neutralized with 1/20th volume 3M Tris pH 9.0. The material was quantitated by measuring absorbance at 280nm and by comassie blue staining of PAGE/SDS/Laemmli gels.

In order to produce non-sulfated forms of soluble PSGL-1, COS cell transfections of the relevant Fc chimeras were performed as described above except that following transfection the cells were cultured in the presence of 50mM Chlorate (Sigma).

Quantitative adhesion of CHO:P-selectin, CHO:E-selectin and CHO:L-selectin expressing cells was performed as described in Example 4(c), with the following modifications: COS cell and antibodies were omitted. Instead, 48-well microtiter plates (Costar) were coated for 16 hours at 4°C with varying quantities of protein A-purified soluble PSGL-1/Fc chimeras. The unbound material was removed and the coated wells were treated with Hank's buffered saline (HBS) with 1mg/ml BSA and 2mM CaCl_2 for 1 hour at 4°C. Tritium labeled CHO selectin expressing cells were added and binding quantitated as described in Example 4(c).

C. Effects of Alteration of N-Linked Glycosylation Sites

Constructs expressing three P-selectin ligand-IgG chimeras were constructed to examine the effects of N-linked glycosylation sites on selectin binding. These constructs had the following characteristics:

- 148.Fc amino acids 42-189 of SEQ ID NO:2
 Q70.148.Fc amino acids 42-189 of SEQ ID NO:2, with
 the asparagine residue at position 111
 of SEQ ID NO:2 replaced with a
 5 glutamine residue
 H24.Q70.148.Fc amino acids 42-189 of SEQ ID NO:2, with
 the asparagine residue at position 65
 of SEQ ID NO:2 with a histidine residue
 and the asparagine residue at position
 10 111 of SEQ ID NO:2 replaced with
 glutamine residue

These constructs are schematically represented in Fig. 6.

The binding of these constructs to protein A and P-selectin-IgG chimera (LEC- γ 1) was compared. The results of
 15 these experiments are shown in Fig. 7. Comparison of lanes
 4, 5 and 6 in the autoradiograph demonstrates that removal
 of one or both of the first two N-linked glycosylation
 sites in soluble P-selectin ligand protein does not
 significantly effect its binding to P-selectin.

20 D. Effects of Tyrosines

Constructs were made to examine the role of tyrosine
 in P-selectin ligand protein binding to selectins by
 alteration of the anionic region of the soluble protein.
 The following constructs were made:

- 25 Δ Y148.Fc amino acids 42-189 of SEQ ID NO:2, with
 amino acids 46-52 deleted
 FFFE.148.Fc amino acids 42-189 of SEQ ID NO:2, with
 the tyrosine residues at positions 46,
 48 and 51 replaced with phenylalanine
 30 residues and the aspartic acid residue
 at position 52 replaced with a glutamic
 acid residue

These constructs are schematically represented in Fig. 8.

The degree and sites of sulfation of P-selectin ligand
 35 protein were examined by expressing relevant constructs in
 the presence of radioactively labelled sulfate. The degree
 of sulfation of 148.Fc and Δ Y.148.Fc were compared to that

of a P-selectin-IgG chimera, which was not sulfated. Results are depicted in Fig. 9. These data demonstrate that the majority of sulfate incorporation is into the anionic region of the P-selectin ligand protein.

5 Additional constructs were made to determine whether the sulfation of the anionic region occurred at the tyrosine residues. The following additional constructs were made:

10	FYYD.19.Fc	amino acids 42-60 of SEQ ID NO:2, with the tyrosine residue at position 46 of SEQ ID NO:2 replaced with a phenylalanine residue
15	FFYD.19.Fc	amino acids 42-60 of SEQ ID NO:2, with the tyrosine residues at positions 46 and 48 of SEQ ID NO:2 replaced with a phenylalanine residues
20	FFFD.19.Fc	amino acids 42-60 of SEQ ID NO:2, with the tyrosine residues at positions 46, 48 and 51 of SEQ ID NO:2 replaced with phenylalanine residues

These constructs are schematically represented in Fig. 9.

25 The degree of sulfation of these constructs was compared to 19.Fc ("YYD.19.Fc"). Results are shown in Fig. 10. FYYD.19.Fc showed significant sulfation while FFFD.19.Fc was substantially less sulfated. Thus, the tyrosine residues of the anionic region of P-selectin
30 ligand proteins are the major site of sulfation.

Removal of sulfate from P-selectin ligand protein substantially reduces its binding to P-selectin. The binding of 148.Fc treated with chlorate to P-selectin was examined. As shown in Fig. 11, inhibition of sulfation by

chlorate treatment substantially reduced the amount of P-selectin ligand protein binding to P-selectin.

E. Effects of C-terminal Deletions

Several additional C-terminal deleted constructs were made as follows:

254.Fc amino acids 42-295 of SEQ ID NO:2

47.Fc amino acids 42-88 of SEQ ID NO:2

19.Fc amino acids 42-60 of SEQ ID NO:2

These constructs are schematically represented in Fig. 12.

The binding of 254.Fc, 148.Fc, 47.Fc and 19.Fc to P-selectin, E-selectin and L-selectin was tested. Figs. 23 and 24 compare the binding of these deletion chimeras to selectins and controls. Results are also summarized in Fig. 12.

F. Binding to P-selectin and E-selectin Expressing Cells

Binding of various constructs described above to cells expressing P-selectin and E-selectin was compared using a quantitative plate binding assay of Example 4(c) (which is schematically described in Fig. 13).

Fig. 14 compares the binding of 148.Fc, Δ Y.148.Fc, FFFE.148.Fc and human IgG1 to P-selectin expressing CHO cells. Deletion of all of the tyrosine residues in the anionic region in Δ Y.148.Fc eliminated binding. Changing the tyrosine residues to phenylalanine residues in FFFE.148.Fc substantially reduced binding as compared to 148.Fc. Thus, it was demonstrated that the presence of the full length anionic region is essential to P-selectin binding and that P-selectin binding is enhanced by sulfation in this region. Fig. 14 also reports control experiments demonstrating that 148.Fc, Δ Y.148.Fc and FFFE.148.Fc do not bind to CHO cells which do not express selectin.

Fig. 15 compares the binding of 148.Fc, Δ Y.148.Fc, FFFE.148.Fc and human IgG1 to E-selectin expressing CHO cells. E-selectin binding was unaffected by the deletions or alterations of the native sequence. Thus, it was

demonstrated that the anionic region is not required for E-selectin binding.

Fig. 16 summarizes the results of Figs. 14 and 15.

Fig. 17 compares the binding of 47.Fc to P- and E-selectin expressing CHO cells. 47.Fc demonstrated substantial binding to both selectins despite deletion of the N-linked glycosylation sites at positions 111 and 292 of SEQ ID NO:2.

Fig. 19 compares the binding of FYYD.19.Fc, FFFD.19.Fc, H24.Q70.148.Fc, 148.Fc, and human IgG1 to P-selectin expressing CHO cells. Replacement of all of the tyrosine residues in the anionic region in FFFD.19.Fc eliminated binding. Changing the tyrosine residue at position 46 to a phenylalanine residue in FYYD.19.Fc substantially reduced binding as compared to 148.Fc. Alteration of the N-linked glycosylation sites in H24.Q70.148.Fc did not affect binding. Thus, it was demonstrated that P-selectin binding is enhanced by sulfation in the anionic region and that N-linked glycosylation is not required for P-selectin binding. Fig. 19 also reports control experiments demonstrating that FYYD.19.Fc, FFFD.19.Fc, H24.Q70.148.Fc and 148.Fc do not bind to CHO cells which do not express selectin more than human IgG1 alone.

Fig. 20 compares the binding of FYYD.19.Fc, FFFD.19.Fc, H24.Q70.148.Fc, 148.Fc, and human IgG1 to E-selectin expressing CHO cells. Truncation of the ligand protein to the degree of FYYD.19.Fc and FFFD.19.Fc substantially reduced E-selectin binding. Alteration of the N-linked glycosylation sites in H24.Q70.148.Fc did not significantly affect E-selectin binding. Thus, it was demonstrated that P-selectin ligand proteins comprising amino acids 42 to 60 of SEQ ID NO:2 can selectively bind P-selectin, and, to a substantially less, extent E-selectin.

Fig. 21 summarizes the results of Figs. 19 and 20.

G. Conclusions Regarding P- and E-Selectin Binding

Although applicants do not wish to be bound by any theory, these data allow several conclusions regarding the relationship between P-selectin binding and E-selectin binding by P-selectin ligand proteins. N-linked carbohydrates are not required for binding of a P-selectin ligand protein to either P- or E-selectin. P-selectin ligand proteins as small as comprising as little as amino acids 42-60 of SEQ ID NO:2 are capable of binding to P-selectin, and, to a substantially less extent E-selectin.

Fig. 22 depicts a proposed schematic model for binding of P-selectin ligand proteins to P- and E-selectin. O-linked sLe^x carbohydrate has been demonstrated to be required for both P- and E-selectin binding. Data presented herein demonstrate that sulfated tyrosine residues are implicated in P-selectin binding, but not E-selectin binding. Applicants' data also suggests that no N-linked glycosylation binding site is required.

20

Example 11

Examination of Aggregation Phenomena and Dimer Formation in Forms of PSGL-1

A panel of PSGL-1 mutants were constructed by site-directed mutagenesis and/or PCR amplification with primers that introduced a stopcodon. The template for all mutagenesis experiments was pPL85.R16 (ATCC 75577, deposited by applicants).

The first group of mutants (C310S and C327S) encode full-length PSGL-1.R16 with only one amino acid change compared to wild-type PSGL-1.R16 (Cys to Ser at position 310 or 327, respectively). COS cells, co-transfected with pEA.3/4FT and the mutants C310S or C327S, were labeled with ³⁵S-methionine. Cell lysates were prepared and the mutant proteins were immunoprecipitated with the P-selectin ligand polyclonal antibody of Example 7(A) and analyzed by SDS-polyacrylamide gel electrophoresis under non-reducing and reducing conditions.

The mutant C327S as well as wild-type PSGL-1.R16 migrated as a homodimer under non-reducing conditions and as a monomer under reducing conditions. In contrast, the mutant C310S migrated as a monomer both under non-reducing and reducing conditions, indicating that the cysteine at position 310 is required for dimer formation of PSGL-1.

Both mutants were also analyzed for their ability to bind to P-selectin. Detergent extracts of co-transfected COS cells were precipitated with the LEC- γ 1 chimera of Example 4(A). The precipitates were analyzed by SDS-PAGE under non-reducing and reducing conditions and by autoradiography. Both PSGL-1.R16 and C327S were efficiently precipitated by LEC-g1, whereas C310S binding to LEC- γ 1 was greatly reduced, indicating that the dimeric form of PSGL-1 binds P-selectin more tightly than the monomeric form.

The second set of mutants encode soluble forms of PSGL-1.R16 and are listed in Table I. The mutant Δ TM was generated by site-directed mutagenesis and has a deletion of the transmembrane domain (amino acids 313-333) followed by RLSRKA. The mutants L311, L312, A313, I314, L315, A318 and T322 were generated by site-directed mutagenesis or PCR amplification with PCR primers that introduced a stop codon in the desired position. The name of the mutant refers to the C-terminal amino acid of each truncated soluble form of PSGL-1.R16. The mutants were analyzed according to the following criteria:

1. Expression and secretion from transfected COS cells
2. Dimer versus monomer formation
3. Lack of aggregate formation
4. P-selectin binding (LEC- γ 1 chimera)

The mutants Δ TM and I316 fulfilled all four criteria. The shorter soluble forms of PSGL-1, such as sPSL.QC of Example 5(A), L311, L312, A313, I314 and L315 did not form dimers as well and the longer soluble forms of PSGL-1, such as sPSL.T7 of Example 5(C), A318 and T322 formed high molecular weight aggregates which were less desirable.

CHO cells, already expressing 3/4 fucosyltransferase and Core2 transferase, were transfected with psPSL.T7, Δ TM, I316 or psPSL.QC and amplified using methotrexate. Stable clones were isolated and labeled with 35 S-methionine.

Conditioned media was either analyzed directly or first precipitated with LEC- γ 1 and then analyzed by SDS-PAGE under non-reducing and reducing conditions (Figure 25). The results indicated that Δ TM and I316 were most efficient in dimer formation and P-selectin binding.

Table I

Mutant	Dimer Formation	High MW Aggregates	P-selectin binding
PSL.QC	+	-	+
L311	+	-	+
L312	-	-	-
A313	-	-	-
I314	-	-	-
L315	+	-	+
I316	++	-	++
A318	++	+	++
T322	++	+	++
Δ TM	++	-	++

Example 12Specificity of PSGL-1 Binding to P- and E-Selectins

Materials. A chimeric protein comprising the extracellular domain of human E-selectin and the Fc portion of human IgG₁ was constructed analogously to the P-selectin chimera, LEC- γ 1, described earlier. The soluble E-selectin chimera was expressed in baculovirus-infected *Trichoplusia* ni high five cells (Invitrogen) and purified to homogeneity by Protein A Sepharose chromatography. Plasmid vectors

pEA.3/4FT, pPL85, pFCD43, and pEA.sPACE, for COS expression of a(1,3/1,4)-fucosyltransferase (Fuc-TIII), PSGL-1, CD43 (leukosialin), and soluble paired basic amino acid converting enzyme (PACE), respectively, have been described herein and in the literature (Sako et al. (1993) *Cell* 75, 1179-1186; Rehemtulla, A. & Kaufman, R. J. (1992) *Curr. Opin. Biotechnol.* 3, 560-565; Wasley et al. (1993) *J. Biol. Chem.* 268, 8458-8465). Fuc-TVII cDNA (plasmid pMT.FT7) was cloned from an HL60 cDNA expression library using oligonucleotide probes derived from the published sequence (Natsuka et al. (1994) *Journal of Biological Chemistry* 269, 16789-16794; Sasaki et al. (1994) *J. Biol. Chem.* 269, 14730-14737). A polyclonal neutralizing rabbit antibody, Rb3443, was raised against a peptide comprising the first 15 amino acids of the mature (PACE-cleaved) N-terminus of PSGL-1. Monoclonal anti-CD43 antibodies from either Becton Dickinson or Biodesign International and isotype control antibodies were coupled to a solid support consisting of Sepharose TM-4E with a covalently attached goat affinity-purified antibody to mouse IgG (Cappel, Organon Teknika Corporation). Affinity coupling of selectin chimeras and murine antibodies to Protein A Sepharose 4 Fast Flow (Pharmacia) and to the anti-mouse IgG resin, respectively, was carried out at a ratio of 2 mg protein/ml of resin. Antiserum Rb3443 was coupled to Protein A Sepharose at 1 ml/ml resin. Coupling efficiencies, indicated by micro-BCA assay (Pierce) of the post-reacted supernatants, were at least 95%. Aprotinin and pepstatin were from Boehringer Mannheim and benzamidine, leupeptin, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma.

Labeling and Membrane Extraction of Myeloid Cells.
U937 or HL60 cells grown in suspension to a density of $\sim 1.3 \times 10^6$ cells/ml were labeled in 50 ml of RPMI1640 medium supplemented with 10% fetal bovine serum and 2.5 mCi of ^3H -glucosamineHCl (Dupont/NEN) for 48 hr. Activities of greater than 1 cpm/cell were routinely obtained by this technique. The labeled cells were washed with PBS,

resuspended in cell lysis buffer (10 mM MOPS, 150 mM NaCl, 4 mM CaCl₂ and 4 mM MgCl₂, pH 7.5 containing protease inhibitors 20 mg/ml aprotinin, 10 mM benzamidine, 20 mg/ml leupeptin, 8 mg/ml pepstatin, and 10 mM PMSF) and subjected to several cycles of probe sonication on ice. Nuclei and cell debris were removed by low speed centrifugation and the cell membranes recovered from the supernatant by centrifugation at 100,000g RCF for 1 hr, washed by resuspension and high speed centrifugation in cell lysis buffer containing 1 M NaCl, and finally resuspended in 3 ml membrane solubilization buffer (cell lysis buffer containing 1% Triton X-100). Several cycles of sonication and incubation on ice were employed to solubilize the membrane fraction. Finally, a low speed centrifugation step was employed to remove insoluble membrane residue.

Labeling and Membrane Extraction of Transfected COS Cells. COS M6 cells were transfected using DEAE-dextran and chloroquine (25) employing 8 µg of plasmids pPL85 or pFCD43 and 4 µg of pEA.SPACE, as well as 4 µg of pEA.3/4FT or PMT.FT7. After 40-45 hr recovery the transfected cells were starved in serum-and methionine-free DME medium for 30 min and then fed [³⁵S]-methionine in serum-free DME for 5 hr. The labeled cells were washed, incubated with EGTA to loosen them from the dish surface, scraped from the dish, pelleted, and suspended in cold 10mM PIPES buffer, pH 7.5, containing 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, and protease inhibitors (see above). Membrane extraction then was carried out by sonication, low speed centrifugation, high speed centrifugation, and solubilization in membrane lysis buffer as above, for labeled myeloid cells.

Affinity Precipitations. Membrane extracts were diluted 1:4 or 1:5 with cell lysis buffer or with TBSC buffer (20 mM TrisHCl, 150 mM NaCl, 2 mM CaCl₂, pH 7.5) supplemented with 5 mg/ml bovine serum albumin (approximately 99%, Sigma). Extracts thus diluted to 0.2-0.25% Triton X-100 were incubated with human IgG₁-Protein A Sepharose with end-over-end mixing at 4°C

overnight. The precleared supernatants then were reacted for 6-12 hrs at 4°C with Protein A Sepharose precoupled with E-or P-selectin chimeras, control human IgG₁, Rb3443 or with rabbit pre-immune serum or with anti-CD43 antibody or isotype control precoupled to goat anti-mouse IgG Sepharose. The resins were washed 5 or more times in buffer containing 0.1-0.5% Triton X-100 until the radioactivity of the wash supernatants was reduced to background level. Elution of proteins bound specifically to P-or E-selectin resins was accomplished with 10 mM EDTA or 5 mM EDTA/5 mM EGTA at room temperature or by boiling in SDS-PAGE sample buffer (Laemmli, U. K. (1970) *Nature* 227, 680-685), whereas elution of proteins bound to antibody resins was achieved exclusively by the latter means. For resolution under reducing conditions, dithiothreitol was added to the sample buffer to a final concentration of 100 mM. Samples thus prepared were resolved by SDS-PAGE on 7.5% gels, treated with En³Hance (Dupont), dried, and exposed to autoradiography film.

For sequential affinity capture experiments, membrane extracts were precleared, affinity precipitated with P-or E-selectin or human IgG₁, and washed as above. Samples then were eluted twice from the resins with 5 mM EDTA in 10 mM MOPS, 150 mM NaCl, pH 7.5 for 1 hr at 4°C with tumbling. The first and second eluates were combined and then immunoprecipitated with immobilized Rb3443 according to the protocols outlined above.

RESULTS:

Soluble E-and P-selectin chimeras were used, in parallel with control human IgG₁, to probe detergent-solubilized membrane extracts of ³H-glucosamine-labeled U937 cells as described under "Methods". Examination of eluates from the immobilized selectins by SDS-PAGE/autoradiography (Fig. 26) revealed the presence in both P-and E-selectin eluates of a major protein species with identical electrophoretic properties: Mr 200-kDa non-reduced with conversion to a species of Mr

120-kDa following reduction (Fig. 26, lanes 2 and 3, respectively). Occasionally, additional bands were observed in both E-and P-selectin eluates presumably corresponding to this major band and reflecting the presence of naturally reduced material (the 120-kDa species in non-reduced samples) and incomplete reduction (the 200-kDa species in reduced samples). Additionally, a trace band of Mr 150-kDa in the E-selectin eluate which was unaffected by reduction with DTT was occasionally observed. No bands were observed in control experiments using immobilized human IgG₁ (Fig. 26, lane 1) or where elution of selectin resins was performed in the absence of EDTA or SDS (data not shown). Essentially identical results were obtained using HL-60 cells (data not shown). Hence, the nature of these recognition events is interpreted to be specific metal-dependant interactions of these proteins with the respective selectins, presumably via the lectin domains (Lasky, L. A. (1992) *Science* 258, 964-969; Drickamer, K. (1988) *J. Biol. Chem.* 263, 9557).

The metal-dependant recognition and electrophoretic behavior of the major band precipitated with E-selectin was consistent with the properties of the previously identified P-selectin counterreceptor, P-selectin glycoprotein ligand or PSGL-1 (Moore et al. (1994) *J. Biol. Chem.* 269, 23318-23327; Moore et al. (1992) *J. Cell Biol.* 118, 445-456; Sako et al.).

To assess whether this species was indeed PSGL-1, EDTA eluates of the both E-and P-selectin precipitates were subsequently reacted with the PSGL-1 specific polyclonal antiserum Rb3443. As shown in Fig. 27, the major band isolated by affinity capture with either selectin was immunoprecipitated using this antiserum (lanes 3 and 4, respectively). No species were detected after immunoprecipitation of the control IgG₁ EDTA eluate (Fig. 27, lane 5). Direct immunoprecipitations using fresh ³H-labeled U937 membrane extracts confirmed the specificity of Rb3443: precipitation with Rb3443 results in the

recovery of a single band with the electrophoretic properties of PSGL-1 (Mr 200-kDa non-reduced, Mr 120-kDa reduced; Fig. 27, lane 2) whereas precipitation with pre-immune antiserum fails to capture any material (Fig. 27, lane 1). These results indicate that the major protein species specifically captured from myeloid cells by both E-and P-selectins is PSGL-1.

To further assess the specificity of E-selectin for PSGL-1, U937 membrane lysates were probed directly for the presence of CD43 (or leukosialin), an abundant cell surface sialoglycoprotein known to bear the major portion of myeloid cell SLe^x residues (Maemura, K. & Fukuda, M. (1992) *J. Biol. Chem.* 267, 24379-24386). Thus, membrane extracts of ³H-glucosamine-labeled U937 cells were probed with an anti-CD43 antibody in parallel with the PSGL-1 specific antiserum Rb3443 and control antibodies as described under "Methods". From identical quantities of membrane lysate, the CD43 antibody precipitated in excess of 30-fold greater radioactive counts than did the PSGL-1 antiserum. Evaluation of the immunoprecipitates by SDS-PAGE/autoradiography (Fig. 28) revealed a single specific band for each antibody. Rb3443 captured a single species with the electrophoretic characteristics of PSGL-1 (Fig. 28, Lane 2). In contrast, the CD43 antibody precipitated a species with an Mr 120-kDa which was insensitive to reduction (Fig. 28, Lane 4), consistent with the absence of cysteine in CD43. Immunoprecipitations with control antibodies (Fig. 28, lanes 1 and 3) proved negative as expected. There appears to be considerably greater quantities of CD43 than PSGL-1 in U937 cells, consistent with the quantitation of these proteins in HL-60 cells (Ushiyama et al. (1993) *J. Biol. Chem.* 268, 15229-15237). Thus, the inability of E-selectin to precipitate CD43 from myeloid cells does not appear to be due to its absence in these cell lines. While we cannot exclude the possibility that E-selectin captures trace quantities of CD43 (ie., the low-intensity Mr 120-kDa band in Fig. 26, non-reduced lane

3 which is also consistent with monomeric PSGL-1), PSGL-1 appears to be the major protein precipitated from myeloid membrane extracts.

Recombinant PSGL-1 expressed in COS cells is best
5 achieved with cotransfection of the PSGL-1 cDNA with a cDNA encoding an $\alpha(1,3/1,4)$ fucosyltransferase (Fuc-TIIII) for P-selectin binding (Sako et al.). Interestingly, initial efforts to demonstrate E-selectin recognition of recombinant PSGL-1 failed: E-selectin was unable to capture
10 the counterreceptor from cotransfected COS cell membrane lysates under conditions where P-selectin capture was successful. One interpretation of this result is that Fuc-TIIII was able to modify recombinant PSGL-1 for recognition by P-selectin but was unable to replicate the
15 appropriate modification(s) found in myeloid PSGL-1 necessary for E-selectin recognition. The recent cloning of a myeloid fucosyltransferase, Fuc-TVII (Natsuka et al. (1994) *Journal of Biological Chemistry* 269, 16789-16794; Sasaki et al. (1994) *J. Biol. Chem.* 269, 14730-14737), that
20 is also capable of generating SLe^x carbohydrate structures, allowed evaluation of this interpretation.

COS cells were cotransfected with cDNAs encoding either PSGL-1 or CD43 and either Fuc-TIIII or Fuc-TVII. Membrane lysates were prepared from the transfected COS
25 cells and these were precipitated with either immobilized E-or P-selectin chimeras or with antibodies to either PSGL-1 or to CD43. The precipitated products were evaluated by SDS-PAGE/autoradiography following their release by EDTA/EGTA (for selectin mediated binding) or by boiling in
30 SDS (for immunoprecipitations). The results are shown in Fig. 29.

As observed in Fig. 29A, E-selectin capture of COS-expressed PSGL-1 was dependant upon the nature of the fucosyltransferase used in the transfection. In three
35 separate experiments, Fuc-TVII, but not Fuc-TIIII, supported PSGL-1 precipitation by E-selectin. The inability of Fuc-TIIII to confer E-selectin reactivity to PSGL-1 cannot

be attributed to a lack of PSGL-1 expression as the specific antiserum Rb3443 immunoprecipitated significant and comparable quantities of PSGL-1 from both Fuc-TIIII and Fuc-TVII transfections (Fig 29C). Furthermore, P-selectin
5 was capable of precipitating equivalent quantities of PSGL-1 with either fucosyltransferase (Fig. 29B), demonstrating that Fuc-TIIII and Fuc-TVII are expressed and active in these cotransfections.

Within the COS recombinant expression system as in
10 myeloid cells, high-affinity E-selectin recognition was also dependent upon the presence of an appropriate polypeptide. Although the polypeptide length, apparent molecular weight, and high frequency and specific types of posttranslational modifications are similar in CD43 and
15 PSGL-1 (Maemura, K. & Fukuda, M. (1992) *J. Biol. Chem.* 267, 24379-24386), neither fucosyltransferase was able to confer high-affinity E-selectin (or P-selectin) recognition to recombinant leukosialin in cotransfected COS cells (Fig 29A). Immunoprecipitations with the anti-CD43 antibody
20 indicate that comparable quantities of leukosialin were expressed in both Fuc-TIIII and Fuc-TVII cotransfections (Fig. 29C). The failure of E-selectin to capture CD43 was not due to lack of fucosyltransferase activity within the cotransfected COS cells. FACS analysis of COS cells
25 transfected with either PSGL-1 or CD43 and either Fuc-TIIII or Fuc-TVII all show high levels of reactivity with the SLeX specific antibody CSLEX-1. Therefore, these results suggest that high-affinity E-selectin recognition requires the presence of a specific polypeptide(s) that is
30 appropriately modified by a specific fucosyltransferase.

Example 13

Inhibition of P-Selectin/PSGL-1 Binding by PSGL-1 Derived Peptides

35 A number of peptides derived from the sequence of PSGL-1 (SEQ ID NO:2) were tested for their ability to

inhibit P-selectin/PSGL-1 binding. The tested peptides are listed in Fig. 30.

Inhibition was tested according to the following protocol. The wells of a 96 well plate were coated overnight at 4°C with PSGL-1 in 50 µl of 10 mM MOPS, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂ at pH 7.5. After removal of the liquid from the wells, 150 µl of 10 mM MOPS, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 0.05% tween-20, 0.05% gelatin at pH 7.5 was added per well to block the unoccupied sites. After 1/2 hr. to 2 hr. the block buffer was removed from the wells and 100 µl of a complex of Lec-γ1 (P-selectin-human IgG Fc chimera) (2 µg/ml), biotinylated goat anti-human antibody, and streptavidin-conjugated alkaline phosphatase (which had been allowed to tumble at room temperature for 30 minutes to 1 hour) plus any potential inhibitors were added per well. Each plate was shaken and rapped to remove the block from plate. The incubation proceeded for 1 hr at room temperature rotating in the dark. The unbound complex was washed off the plate with two 150 µl portions of 10 mM MOPS, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 0.05% tween-20 followed by 150 µl of 1M diethanolamine, 0.5 mM MgCl₂. The chromogenic substrate for alkaline phosphatase, PNPP, in 10 mM DEA/0.5 mM MgCl₂ was added and the plate is then read at 405 nm.

The results of these assays are reported in Fig. 30. The peptides comprising amino acids 48-51 (in which the tyrosine residues have been phosphorylated) and amino acids 42-56 of SEQ ID NO:2 provided particularly desirable results.

30

Example 14

Purification of a Soluble Form of PSGL-1

Substantial purification of a soluble form of P-selectin ligand protein has been achieved according to the protocol described below.

35

A soluble P-selectin ligand protein, I316 (amino acid 42 to amino acid 316 of SEQ ID NO:2) was expressed in CHO

cells as described herein. CHO cell conditioned media was concentrated with a Pellicon ultrafiltration membrane unit (Millipore) with either 10,000 molecular weight cutoff (MWCO) or 30,000 MWCO to about 10 times the original concentration. The buffer was then exchanged into 25 mM Tris, 1mM CaCl₂, pH 7.4.

The buffer-exchanged concentrate was loaded onto a Toyopearl QAE 550C (TosoHaas) column. Alternatively, the buffer exchange step can be omitted and the concentrate can be diluted one part concentrate to three parts 25 mM Tris, 1mM CaCl₂, pH 7.4, and then loaded onto the column. The column was washed with 5-10 column volumes (CV) of 25 mM Tris, 1mM CaCl₂, pH 7.4 at 4°C.

The P-selectin ligand protein eluted from the column with a linear NaCl gradient (0 M NaCl to 1.0 M NaCl) in the 25 mM Tris, 1mM CaCl₂, pH 7.4 buffer in approximately five column volumes. Two peaks were eluted from the column. The second peak contained the P-selectin ligand protein and was collected in bulk.

The peak from the QAE column was concentrated with a tangential flow ultrafiltration membrane (Millipore) with a 30,000 MWCO and was then buffer exchanged into 25 mM Tris, 150 mM NaCl, 1mM CaCl₂, pH 7.4 at 4°C.

The buffer exchanged concentrate was loaded onto a Jacalin Agarose column overnight at 4°C. The column was washed with the diafiltration buffer and the P-selectin ligand protein was eluted with a gradient of methyl α -D-galactopyranoside (0-100 mM or)-50 mM methyl α -D-galactopyranoside) at 20°C. Fractions from the Jacalin column were analyzed by SDS-PAGE and the purest fractions were pooled.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: GENETICS INSTITUTE, INC.
- (ii) TITLE OF INVENTION: NOVEL P-SELECTIN LIGAND PROTEIN
- (iii) NUMBER OF SEQUENCES: 34
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Legal Affairs
 - (B) STREET: 87 CambridgePark Drive
 - (C) CITY: Cambridge
 - (D) STATE: MA
 - (E) COUNTRY: USA
 - (F) ZIP: 02140
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/965,662
 - (B) FILING DATE: 23-OCT-1992
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/112,608
 - (B) FILING DATE: 26-AUG-1993
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: PCT/US93/10168
 - (B) FILING DATE: 22-OCT-1993
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/235,398
 - (B) FILING DATE: 28-APR-1994
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/316,305
 - (B) FILING DATE: 30-SEP-1994
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: BROWN, SCOTT A.
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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1649 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens
 (G) CELL TYPE: Promyelocyte
 (H) CELL LINE: HL60
- (vii) IMMEDIATE SOURCE:
 (B) CLONE: PMT21:PL85
- (ix) FEATURE:
 (A) NAME/KEY: 5'UTR
 (B) LOCATION: 1..59
- (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 60..1268
- (ix) FEATURE:
 (A) NAME/KEY: 3'UTR
 (B) LOCATION: 1269..1649

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

GCCACTTCTT CTGGGCCAC GAGGCAGCTG TCCCATGCTC TGCTGAGCAC GGTGGTGCC      59
ATG CCT CTG CAA CTC CTC CTG TTG CTG ATC CTA CTG GGC CCT GGC AAC      107
Met Pro Leu Gln Leu Leu Leu Leu Ile Leu Leu Gly Pro Gly Asn
  1                    5                      10              15

AGC TTG CAG CTG TGG GAC ACC TGG GCA GAT GAA GCC GAG AAA GCC TTG      155
Ser Leu Gln Leu Trp Asp Thr Trp Ala Asp Glu Ala Glu Lys Ala Leu
                20                      25              30

GGT CCC CTG CTT GCC CGG GAC CGG AGA CAG GCC ACC GAA TAT GAG TAC      203
Gly Pro Leu Leu Ala Arg Asp Arg Arg Gln Ala Thr Glu Tyr Glu Tyr
          35                      40              45

CTA GAT TAT GAT TTC CTG CCA GAA ACG GAG CCT CCA GAA ATG CTG AGG      251
Leu Asp Tyr Asp Phe Leu Pro Glu Thr Glu Pro Pro Glu Met Leu Arg
    50                      55              60

AAC AGC ACT GAC ACC ACT CCT CTG ACT GGG CCT GGA ACC CCT GAG TCT      299
Asn Ser Thr Asp Thr Thr Pro Leu Thr Gly Pro Gly Thr Pro Glu Ser
  65                      70              75              80

ACC ACT GTG GAG CCT GCT GCA AGG CGT TCT ACT GGC CTG GAT GCA GGA      347
Thr Thr Val Glu Pro Ala Ala Arg Arg Ser Thr Gly Leu Asp Ala Gly
                85                      90              95

GGG GCA GTC ACA GAG CTG ACC ACG GAG CTG GCC AAC ATG GGG AAC CTG      395
Gly Ala Val Thr Glu Leu Thr Thr Glu Leu Ala Asn Met Gly Asn Leu
          100                      105              110

TCC ACG GAT TCA GCA GCT ATG GAG ATA CAG ACC ACT CAA CCA GCA GCC      443
Ser Thr Asp Ser Ala Ala Met Glu Ile Gln Thr Thr Gln Pro Ala Ala
    115                      120              125

ACG GAG GCA CAG ACC ACT CCA CTG GCA GCC ACA GAG GCA CAG ACA ACT      491
Thr Glu Ala Gln Thr Thr Pro Leu Ala Ala Thr Glu Ala Gln Thr Thr
    130                      135              140

CGA CTG ACG GCC ACG GAG GCA CAG ACC ACT CCA CTG GCA GCC ACA GAG      539
Arg Leu Thr Ala Thr Glu Ala Gln Thr Thr Pro Leu Ala Ala Thr Glu
  145                      150              155              160

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GCA CAG ACC ACT CCA CCA GCA GCC ACG GAA GCA CAG ACC ACT CAA CCC Ala Gln Thr Thr Pro Pro Ala Ala Thr Glu Ala Gln Thr Thr Gln Pro 165 170 175	587
ACA GGC CTG GAG GCA CAG ACC ACT GCA CCA GCA GCC ATG GAG GCA CAG Thr Gly Leu Glu Ala Gln Thr Thr Ala Pro Ala Ala Met Glu Ala Gln 180 185 190	635
ACC ACT GCA CCA GCA GCC ATG GAA GCA CAG ACC ACT CCA CCA GCA GCC Thr Thr Ala Pro Ala Ala Met Glu Ala Gln Thr Thr Pro Pro Ala Ala 195 200 205	683
ATG GAG GCA CAG ACC ACT CAA ACC ACA GCC ATG GAG GCA CAG ACC ACT Met Glu Ala Gln Thr Thr Gln Thr Thr Ala Met Glu Ala Gln Thr Thr 210 215 220	731
GCA CCA GAA GCC ACG GAG GCA CAG ACC ACT CAA CCC ACA GCC ACG GAG Ala Pro Glu Ala Thr Glu Ala Gln Thr Thr Gln Pro Thr Ala Thr Glu 225 230 235 240	779
GCA CAG ACC ACT CCA CTG GCA GCC ATG GAG GCC CTG TCC ACA GAA CCC Ala Gln Thr Thr Pro Leu Ala Ala Met Glu Ala Leu Ser Thr Glu Pro 245 250 255	827
AGT GCC ACA GAG GCC CTG TCC ATG GAA CCT ACT ACC AAA AGA GGT CTG Ser Ala Thr Glu Ala Leu Ser Met Glu Pro Thr Thr Lys Arg Gly Leu 260 265 270	875
TTC ATA CCC TTT TCT GTG TCC TCT GTT ACT CAC AAG GGC ATT CCC ATG Phe Ile Pro Phe Ser Val Ser Ser Val Thr His Lys Gly Ile Pro Met 275 280 285	923
GCA GCC AGC AAT TTG TCC GTC AAC TAC CCA GTG GGG GCC CCA GAC CAC Ala Ala Ser Asn Leu Ser Val Asn Tyr Pro Val Gly Ala Pro Asp His 290 295 300	971
ATC TCT GTG AAG CAG TGC CTG CTG GCC ATC CTA ATC TTG GCG CTG GTG Ile Ser Val Lys Gln Cys Leu Leu Ala Ile Leu Ile Leu Ala Leu Val 305 310 315 320	1019
GCC ACT ATC TTC TTC GTG TGC ACT GTG GTG CTG GCG GTC CGC CTC TCC Ala Thr Ile Phe Phe Val Cys Thr Val Val Leu Ala Val Arg Leu Ser 325 330 335	1067
CGC AAG GGC CAC ATG TAC CCC GTG CGT AAT TAC TCC CCC ACC GAG ATG Arg Lys Gly His Met Tyr Pro Val Arg Asn Tyr Ser Pro Thr Glu Met 340 345 350	1115
GTC TGC ATC TCA TCC CTG TTG CCT GAT GGG GGT GAG GGG CCC TCT GCC Val Cys Ile Ser Ser Leu Leu Pro Asp Gly Gly Glu Gly Pro Ser Ala 355 360 365	1163
ACA GCC AAT GGG GGC CTG TCC AAG GCC AAG AGC CCG GGC CTG ACG CCA Thr Ala Asn Gly Gly Leu Ser Lys Ala Lys Ser Pro Gly Leu Thr Pro 370 375 380	1211
GAG CCC AGG GAG GAC CGT GAG GGG GAT GAC CTC ACC CTG CAC AGC TTC Glu Pro Arg Glu Asp Arg Glu Gly Asp Asp Leu Thr Leu His Ser Phe 385 390 395 400	1259
CTC CCT TAGCTCACTC TGCCATCTGT TTTGGCAAGA CCCACCTCC ACGGGCTCTC Leu Pro	1315
CTGGGCCACC CCTGAGTGCC CAGACCCCAA TCCACAGCTC TGGGCTTCCT CGGAGACCCC	1375
TGGGGATGGG GATCTTCAGG GAAGGAATC TGGCCACCCA AACAGGACAA GAGCAGCCTG	1435

GGGCAAGCA GACGGGCAAG TGGAGCCACC TCTTCTCTCC CTCCGCGGAT GAAGCCCAGC 1495
CACATTTTCAG CCGAGGTCCA AGGCAGGAGG CCATTTACTT GAGACAGATT CTCTCCTTTT 1555
TCCTGTCCCC CATCTTCTCT GGGTCCCTCT AACATCTCCC ATGGCTCTCC CCGCTTCTCC 1615
TGGTCACTGG AGTCTCCTCC CCATGTACCC AAGG 1649

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 402 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Pro Leu Gln Leu Leu Leu Leu Ile Leu Leu Gly Pro Gly Asn
1 5 10 15
Ser Leu Gln Leu Trp Asp Thr Trp Ala Asp Glu Ala Glu Lys Ala Leu
20 25 30
Gly Pro Leu Leu Ala Arg Asp Arg Arg Gln Ala Thr Glu Tyr Glu Tyr
35 40 45
Leu Asp Tyr Asp Phe Leu Pro Glu Thr Glu Pro Pro Glu Met Leu Arg
50 55 60
Asn Ser Thr Asp Thr Thr Pro Leu Thr Gly Pro Gly Thr Pro Glu Ser
65 70 75 80
Thr Thr Val Glu Pro Ala Ala Arg Arg Ser Thr Gly Leu Asp Ala Gly
85 90 95
Gly Ala Val Thr Glu Leu Thr Thr Glu Leu Ala Asn Met Gly Asn Leu
100 105 110
Ser Thr Asp Ser Ala Ala Met Glu Ile Gln Thr Thr Gln Pro Ala Ala
115 120 125
Thr Glu Ala Gln Thr Thr Pro Leu Ala Ala Thr Glu Ala Gln Thr Thr
130 135 140
Arg Leu Thr Ala Thr Glu Ala Gln Thr Thr Pro Leu Ala Ala Thr Glu
145 150 155 160
Ala Gln Thr Thr Pro Pro Ala Ala Thr Glu Ala Gln Thr Thr Gln Pro
165 170 175
Thr Gly Leu Glu Ala Gln Thr Thr Ala Pro Ala Ala Met Glu Ala Gln
180 185 190
Thr Thr Ala Pro Ala Ala Met Glu Ala Gln Thr Thr Pro Pro Ala Ala
195 200 205
Met Glu Ala Gln Thr Thr Gln Thr Thr Ala Met Glu Ala Gln Thr Thr
210 215 220
Ala Pro Glu Ala Thr Glu Ala Gln Thr Thr Gln Pro Thr Ala Thr Glu
225 230 235 240
Ala Gln Thr Thr Pro Leu Ala Ala Met Glu Ala Leu Ser Thr Glu Pro
245 250 255

Ser Ala Thr Glu Ala Leu Ser Met Glu Pro Thr Thr Lys Arg Gly Leu
 260 265 270

Phe Ile Pro Phe Ser Val Ser Ser Val Thr His Lys Gly Ile Pro Met
 275 280 285

Ala Ala Ser Asn Leu Ser Val Asn Tyr Pro Val Gly Ala Pro Asp His
 290 295 300

Ile Ser Val Lys Gln Cys Leu Leu Ala Ile Leu Ile Leu Ala Leu Val
 305 310 315 320

Ala Thr Ile Phe Phe Val Cys Thr Val Val Leu Ala Val Arg Leu Ser
 325 330 335

Arg Lys Gly His Met Tyr Pro Val Arg Asn Tyr Ser Pro Thr Glu Met
 340 345 350

Val Cys Ile Ser Ser Leu Leu Pro Asp Gly Gly Glu Gly Pro Ser Ala
 355 360 365

Thr Ala Asn Gly Gly Leu Ser Lys Ala Lys Ser Pro Gly Leu Thr Pro
 370 375 380

Glu Pro Arg Glu Asp Arg Glu Gly Asp Asp Leu Thr Leu His Ser Phe
 385 390 395 400

Leu Pro

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1239 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA (synthetic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens
- (G) CELL TYPE: placenta

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1239

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATG CCT CTG CAA CTC CTC CTG TTG CTG ATC CTA CTG GGC CCT GGC AAC	48
Met Pro Leu Gln Leu Leu Leu Leu Ile Leu Leu Gly Pro Gly Asn	
1 5 10 15	
AGC TTG CAG CTG TGG GAC ACC TGG GCA GAT GAA GCC GAG AAA GCC TTG	96
Ser Leu Gln Leu Trp Asp Thr Trp Ala Asp Glu Ala Glu Lys Ala Leu	
20 25 30	
GGT CCC CTG CTT GCC CGG GAC CGG AGA CAG GCC ACC GAA TAT GAG TAC	144
Gly Pro Leu Leu Ala Arg Asp Arg Arg Gln Ala Thr Glu Tyr Glu Tyr	
35 40 45	
CTA GAT TAT GAT TTC CTG CCA GAA ACG GAG CCT CCA GAA ATG CTG AGG	192
Leu Asp Tyr Asp Phe Leu Pro Glu Thr Glu Pro Pro Glu Met Leu Arg	
50 55 60	

AAC Asn 65	AGC Ser	ACT Thr	GAC Asp	ACC Thr	ACT Thr	CCT Pro	CTG Leu	ACT Thr	GGG Gly	CCT Pro	GGA Gly	ACC Thr	CCT Pro	GAG Glu	TCT Ser	240
ACC Thr	ACT Thr	GTG Val	GAG Glu	CCT Pro	GCT Ala	GCA Ala	AGG Arg	CGT Arg	TCT Ser	ACT Thr	GGC Gly	CTG Leu	GAT Asp	GCA Ala	GGA Gly	288
GGG Gly	GCA Ala	GTC Val	ACA Thr	GAG Glu	CTG Leu	ACC Thr	ACG Thr	GAG Glu	CTG Leu	GCC Ala	AAC Asn	ATG Met	GGG Gly	AAC Asn	CTG Leu	336
TCC Ser	ACG Thr	GAT Asp	TCA Ser	GCA Ala	GCT Ala	ATG Met	GAG Glu	ATA Ile	CAG Gln	ACC Thr	ACT Thr	CAA Gln	CCA Pro	GCA Ala	GCC Ala	384
ACG Thr	GAG Glu	GCA Ala	CAG Gln	ACC Thr	ACT Thr	CAA Gln	CCA Pro	GTG Val	CCC Pro	ACG Thr	GAG Glu	GCA Ala	CAG Gln	ACC Thr	ACT Thr	432
CCA Pro	CTG Leu	GCA Ala	GCC Ala	ACA Thr	GAG Glu	GCA Ala	CAG Gln	ACA Thr	ACT Thr	CGA Arg	CTG Leu	ACG Thr	GCC Ala	ACG Thr	GAG Glu	480
GCA Ala	CAG Gln	ACC Thr	ACT Thr	CCA Pro	CTG Leu	GCA Ala	GCC Ala	ACA Thr	GAG Glu	GCA Ala	CAG Gln	ACC Thr	ACT Thr	CCA Pro	CCA Pro	528
GCA Ala	GCC Ala	ACG Thr	GAA Glu	GCA Ala	CAG Gln	ACC Thr	ACT Thr	CAA Gln	CCC Pro	ACA Thr	GGC Gly	CTG Leu	GAG Glu	GCA Ala	CAG Gln	576
ACC Thr	ACT Thr	GCA Ala	CCA Pro	GCA Ala	GCC Ala	ATG Met	GAG Glu	GCA Ala	CAG Gln	ACC Thr	ACT Thr	GCA Ala	CCA Pro	GCA Ala	GCC Ala	624
ATG Met	GAA Glu	GCA Ala	CAG Gln	ACC Thr	ACT Thr	CCA Pro	CCA Pro	GCA Ala	GCC Ala	ATG Met	GAG Glu	GCA Ala	CAG Gln	ACC Thr	ACT Thr	672
CAA Gln	ACC Thr	ACA Thr	GCC Ala	ATG Met	GAG Glu	GCA Ala	CAG Gln	ACC Thr	ACT Thr	GCA Ala	CCA Pro	GAA Glu	GCC Ala	ACG Thr	GAG Glu	720
GCA Ala	CAG Gln	ACC Thr	ACT Thr	CAA Gln	CCC Pro	ACA Thr	GCC Ala	ACG Thr	GAG Glu	GCA Ala	CAG Gln	ACC Thr	ACT Thr	CCA Pro	CTG Leu	768
GCA Ala	GCC Ala	ATG Met	GAG Glu	GCC Ala	CTG Leu	TCC Ser	ACA Thr	GAA Glu	CCC Pro	AGT Ser	GCC Ala	ACA Thr	GAG Glu	GCC Ala	CTG Leu	816
TCC Ser	ATG Met	GAA Glu	CCT Pro	ACT Thr	ACC Thr	AAA Lys	AGA Arg	GGT Gly	CTG Leu	TTC Phe	ATA Ile	CCC Pro	TTT Phe	TCT Ser	GTG Val	864
TCC Ser	TCT Ser	GTT Val	ACT Thr	CAC His	AAG Lys	GGC Gly	ATT Ile	CCC Pro	ATG Met	GCA Ala	GCC Ala	AGC Ser	AAT Asn	TTG Leu	TCC Ser	912
GTC Val	AAC Asn	TAC Tyr	CCA Pro	GTG Val	GGG Gly	GCC Ala	CCA Pro	GAC Asp	CAC His	ATC Ile	TCT Ser	GTG Val	AAG Lys	CAG Gln	TGC Cys	960
CTG Leu	CTG Leu	GCC Ala	ATC Ile	CTA Leu	ATC Ile	TTG Leu	GCG Ala	CTG Leu	GTG Val	GCC Ala	ACT Thr	ATC Ile	TTC Phe	TTC Phe	GTG Val	1008

TGC ACT GTG GTG CTG GCG GTC CGC CTC TCC CGC AAG GGC CAC ATG TAC	1056
Cys Thr Val Val Leu Ala Val Arg Leu Ser Arg Lys Gly His Met Tyr	
340 345 350	
CCC GTG CGT AAT TAC TCC CCC ACC GAG ATG GTC TGC ATC TCA TCC CTG	1104
Pro Val Arg Asn Tyr Ser Pro Thr Glu Met Val Cys Ile Ser Ser Leu	
355 360 365	
TTG CCT GAT GGG GGT GAG GGG CCC TCT GCC ACA GCC AAT GGG GGC CTG	1152
Leu Pro Asp Gly Gly Glu Gly Pro Ser Ala Thr Ala Asn Gly Gly Leu	
370 375 380	
TCC AAG GCC AAG AGC CCG GGC CTG ACG CCA GAG CCC AGG GAG GAC CGT	1200
Ser Lys Ala Lys Ser Pro Gly Leu Thr Pro Glu Pro Arg Glu Asp Arg	
385 390 395 400	
GAG GGG GAT GAC CTC ACC CTG CAC AGC TTC CTC CCT TAG	1239
Glu Gly Asp Asp Leu Thr Leu His Ser Phe Leu Pro	
405 410	

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 412 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met	Pro	Leu	Gln	Leu	Leu	Leu	Leu	Ile	Leu	Leu	Gly	Pro	Gly	Asn	
1				5				10					15		
Ser	Leu	Gln	Leu	Trp	Asp	Thr	Trp	Ala	Asp	Glu	Ala	Glu	Lys	Ala	Leu
			20					25					30		
Gly	Pro	Leu	Leu	Ala	Arg	Asp	Arg	Arg	Gln	Ala	Thr	Glu	Tyr	Glu	Tyr
		35					40					45			
Leu	Asp	Tyr	Asp	Phe	Leu	Pro	Glu	Thr	Glu	Pro	Pro	Glu	Met	Leu	Arg
	50					55					60				
Asn	Ser	Thr	Asp	Thr	Thr	Pro	Leu	Thr	Gly	Pro	Gly	Thr	Pro	Glu	Ser
65					70					75					80
Thr	Thr	Val	Glu	Pro	Ala	Ala	Arg	Arg	Ser	Thr	Gly	Leu	Asp	Ala	Gly
				85					90					95	
Gly	Ala	Val	Thr	Glu	Leu	Thr	Thr	Glu	Leu	Ala	Asn	Met	Gly	Asn	Leu
			100					105					110		
Ser	Thr	Asp	Ser	Ala	Ala	Met	Glu	Ile	Gln	Thr	Thr	Gln	Pro	Ala	Ala
		115				120						125			
Thr	Glu	Ala	Gln	Thr	Thr	Gln	Pro	Val	Pro	Thr	Glu	Ala	Gln	Thr	Thr
	130					135					140				
Pro	Leu	Ala	Ala	Thr	Glu	Ala	Gln	Thr	Thr	Arg	Leu	Thr	Ala	Thr	Glu
145					150					155					160
Ala	Gln	Thr	Thr	Pro	Leu	Ala	Ala	Thr	Glu	Ala	Gln	Thr	Thr	Pro	Pro
				165					170					175	
Ala	Ala	Thr	Glu	Ala	Gln	Thr	Thr	Gln	Pro	Thr	Gly	Leu	Glu	Ala	Gln
			180					185					190		

Thr Thr Ala Pro Ala Ala Met Glu Ala Gln Thr Thr Ala Pro Ala Ala
 195 200 205
 Met Glu Ala Gln Thr Thr Pro Pro Ala Ala Met Glu Ala Gln Thr Thr
 210 215 220
 Gln Thr Thr Ala Met Glu Ala Gln Thr Thr Ala Pro Glu Ala Thr Glu
 225 230 235 240
 Ala Gln Thr Thr Gln Pro Thr Ala Thr Glu Ala Gln Thr Thr Pro Leu
 245 250 255
 Ala Ala Met Glu Ala Leu Ser Thr Glu Pro Ser Ala Thr Glu Ala Leu
 260 265 270
 Ser Met Glu Pro Thr Thr Lys Arg Gly Leu Phe Ile Pro Phe Ser Val
 275 280 285
 Ser Ser Val Thr His Lys Gly Ile Pro Met Ala Ala Ser Asn Leu Ser
 290 295 300
 Val Asn Tyr Pro Val Gly Ala Pro Asp His Ile Ser Val Lys Gln Cys
 305 310 315 320
 Leu Leu Ala Ile Leu Ile Leu Ala Leu Val Ala Thr Ile Phe Phe Val
 325 330 335
 Cys Thr Val Val Leu Ala Val Arg Leu Ser Arg Lys Gly His Met Tyr
 340 345 350
 Pro Val Arg Asn Tyr Ser Pro Thr Glu Met Val Cys Ile Ser Ser Leu
 355 360 365
 Leu Pro Asp Gly Gly Glu Gly Pro Ser Ala Thr Ala Asn Gly Gly Leu
 370 375 380
 Ser Lys Ala Lys Ser Pro Gly Leu Thr Pro Glu Pro Arg Glu Asp Arg
 385 390 395 400
 Glu Gly Asp Asp Leu Thr Leu His Ser Phe Leu Pro
 405 410

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 2151 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:

- (B) CLONE: pacesol

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATGGAGCTGA GGCCCTGGTT GCTATGGGTG GTAGCAGCAA CAGGAACCTT GGTCCTGCTA	60
GCAGCTGATG CTCAGGGCCA GAAGGTCTTC ACCAACACGT GGGCTGTGCG CATCCCTGGA	120
GGCCAGCGG TGGCCAACAG TGTGGCACGG AAGCATGGGT TCCTCAACCT GGGCCAGATC	180
TTCGGGGACT ATTACCACTT CTGGCATCGA GGAGTGACGA AGCGGTCCCT GTCGCCTCAC	240

CGCCCGCGGC ACAGCCGGCT GCAGAGGGAG CCTCAAGTAC AGTGGCTGGA ACAGCAGGTG	300
GCAAAGCGAC GGAATAACG GGACGTGTAC CAGGAGCCCA CAGACCCCAA GTTTCCTCAG	360
CAGTGGTACC TGTCTGGTGT CACTCAGCGG GACCTGAATG TGAAGGCGGC CTGGGCGCAG	420
GGCTACACAG GGCACGGCAT TGTGGTCTCC ATTCTGGACG ATGGCATCGA GAAGAACCAC	480
CCGGACTTGG CAGGCAATTA TGATCCTGGG GCCAGTTTTG ATGTCAATGA CCAGGACCCT	540
GACCCCCAGC CTCGGTACAC ACAGATGAAT GACAACAGGC ACGGCACACG GTGTGCGGGG	600
GAAGTGGCTG CCGTGGCCAA CAACGGGTGTC TGTGGTGTAG GTGTGGCCTA CAACGCCCCG	660
ATTGGAGGGG TGCGCATGCT GGATGGCGAG GTGACAGATG CAGTGGAGGC ACGCTCGCTG	720
GGCCTGAACC CCAACCACAT CCACATCTAC AGTGCCAGCT GGGGCCCCGA GGATGACGGC	780
AAGACAGTGG ATGGGCCAGC CCGCCTCGCC GAGGAGGCCT TCTTCCGTGG GGTTAGCCAG	840
GGCCGAGGGG GGCTGGGCTC CATCTTTGTC TGGGCCTCGG GGAACGGGGG' CCGGGAACAT	900
GACAGCTGCA ACTGCGACGG CTACACCAAC AGTATCTACA CGCTGTCCAT CAGCAGCGCC	960
ACGCAGTTTG GCAACGTGCC GTGGTACAGC GAGGCCTGCT CGTCCACACT GGCCACGACC	1020
TACAGCAGTG GCAACCAGAA TGAGAAGCAG ATCGTGACGA CTGACTTGCG GCAGAAGTGC	1080
ACGGAGTCTC ACACGGGCAC CTCAGCCTCT GCCCCTTAG CAGCCGGCAT CATTGCTCTC	1140
ACCCTGGAGG CCAATAAGAA CCTCACATGG CGGGACATGC AACACCTGGT GGTACAGACC	1200
TCGAAGCCAG CCCACCTCAA TGCCAACGAC TGGGCCACCA ATGGTGTGGG CCGGAAAGTG	1260
AGCCACTCAT ATGGCTACGG GCTTTTGGAC GCAGGCGCCA TGGTGGCCCT GGCCCAGAAT	1320
TGGACCACAG TGGCCCCCA GCGGAAGTGC ATCATCGACA TCCTCACCGA GCCCCAAGAC	1380
ATCGGGAAAC GGCTCGAGGT GCGGAAGACC GTGACCGCGT GCCTGGGCGA GCCCAACCAC	1440
ATCACTCGGC TGGAGCACGC TCAGGCGCGG CTCACCCTGT CCTATAATCG CCGTGGCGAC	1500
CTGGCCATCC ACCTGGTCAG CCCCATGGGC ACCCGCTCCA CCCTGCTGGC AGCCAGGCCA	1560
CATGACTACT CCGCAGATGG GTTTAATGAC TGGGCCTTCA TGACAACTCA TTCCTGGGAT	1620
GAGGATCCCT CTGGCGAGTG GGTCCTAGAG ATTGAAAACA CCAGCGAAGC CAACAACTAT	1680
GGGACGCTGA CCAAGTTCAC CTCGTAATC TATGGCACCG CCCCTGAGGG GCTGCCCGTA	1740
CCTCCAGAAA GCAGTGGCTG CAAGACCCTC ACGTCCAGTC AGGCCTGTGT GGTGTGCGAG	1800
GAAGGCTTCT CCCTGCACCA GAAGAGCTGT GTCCAGCACT GCCCTCCAGG CTTCGCCCCC	1860
CAAGTCCTCG ATACGCACTA TAGCACCGAG AATGACGTGG AGACCATCCG GGCCAGCGTC	1920
TGCGCCCCCT GCCACGCCTC ATGTGCCACA TGCCAGGGGC CGGCCCTGAC AGACTGCCTC	1980
AGCTGCCCCA GCCACGCCTC CTTGGACCCT GTGGAGCAGA CTTGCTCCCG GCAAAGCCAG	2040
AGCAGCCGAG AGTCCCCGCC ACAGCAGCAG CCACCTCGGC TGCCCCCGGA GGTGGAGGCG	2100
GGGCAACGGC TGCGGGCAGG GCTGCTGCCC TCACACCTGC CTGAGTGATG A	2151

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1587 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(vii) IMMEDIATE SOURCE:

(B) CLONE: sPSL.Fc

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATGCCTCTGC AACTCCTCCT GTTGCTGATC CTACTGGGCC CTGGCAACAG CTTGCAGCTG	60
TGGGACACCT GGGCAGATGA AGCCGAGAAA GCCTTGGGTC CCCTGCTTGC CCGGGACCGG	120
AGACAGGCCA CCGAATATGA GTACCTAGAT TATGATTTCC TGCCAGAAAC GGAGCCTCCA	180
GAAATGCTGA GGAACAGCAC TGACACCACT CCTCTGACTG GGCCTGGAAC CCCTGAGTCT	240
ACCACTGTGG AGCCTGCTGC AAGGCGTTCT ACTGGCCTGG ATGCAGGAGG GGCAGTCACA	300
GAGCTGACCA CGGAGCTGGC CAACATGGGG AACCTGTCCA CGGATTCAGC AGCTATGGAG	360
ATACAGACCA CTCAACCAGC AGCCACGGAG GCACAGACCA CTCCACTGGC AGCCACAGAG	420
GCACAGACAA CTCGACTGAC GGCCACGGAG GCACAGACCA CTCCACTGGC AGCCACAGAG	480
GCACAGACCA CTCCACCAGC AGCCACGGAA GCACAGACCA CTCAACCCAC AGGCCTGGAG	540
GCACAGACCA CTGCACCAGC AGCCATGGAG GCACAGACCA CTGCACCAGC AGCCATGGAA	600
GCACAGACCA CTCCACCAGC AGCCATGGAG GCACAGACCA CTCAACCCAC AGCCATGGAG	660
GCACAGACCA CTGCACCAGA AGCCACGGAG GCACAGACCA CTCAACCCAC AGCCACGGAG	720
GCACAGACCA CTCCACTGGC AGCCATGGAG GCCCTGTCCA CAGAACCCAG TGCCACAGAG	780
GCCCTGTCCA TGGAACTTAC TACCAAAAGA GGTCTGTTCA TACCCTTTTC TGTGTCCTCT	840
GTTACTCACA AGGGCATTCC CATGGCAGCC AGCAATTTGT CCGTCCTGCG GCCGCAGTCT	900
AGAGACAAAA CTCACACATG CCCACCGTGC CCAGCACCTG AACTCCTGGG GGGACCGTCA	960
GTCTTCCTCT TCCCCCAAA ACCCAAGGAC ACCCTCATGA TCTCCCGGAC CCCTGAGGTC	1020
ACATGCGTGG TGGTGGACGT GAGCCACGAA GACCCTGAGG TCAAGTTCAA CTGGTACGTG	1080
GACGGCGTGG AGGTGCATAA TGCCAAGACA AAGCCGCGGG AGGAGCAGTA CAACAGCACG	1140
TACCGTGTGG TCAGCGTCCT CACCGTCCTG CACCAGGACT GGCTGAATGG CAAGGAGTAC	1200
AAGTGCAAGG TCTCCAACAA AGCCCTCCCA GTCCCCATCG AGAAAACCAT CTCCAAAGCC	1260
AAAGGGCAGC CCCGAGAACC ACAGGTGTAC ACCCTGCCCC CATCCCGGGA GGAGATGACC	1320
AAGAACCAGG TCAGCCTGAC CTGCCTGGTC AAAGGCTTCT ATCCCAGCGA CATCGCCGTG	1380
GAGTGGGAGA GCAATGGGCA GCCGGAGAAC AACTACAAGA CCACGCCTCC CGTGCTGGAC	1440
TCCGACGGCT CCTTCTTCCT CTATAGCAAG CTCACCGTGG ACAAGAGCAG GTGGCAGCAG	1500
GGGAACGTCT TCTCATGCTC CGTGATGCAT GAGGCTCTGC ACAACCACTA CACGCAGAAG	1560

AGCCTCTCCC TGTCOC CGGG TAAATAG

1587

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AATTCGTCG ACTCTAGAG

19

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTCTAGAGTC GACGG

15

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 43 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TAGCATACGC TCTAGAGCAT GGATCCCCTG GGTGCAGCCA AGC

43

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCGGAATTCT CAGGTGAACC AAGCCGC

27

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AAGTATCTGT CCAGGGCTTC CAGGT

25

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 48 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AACTACCCAG TGGGAGCACC AGACCACATC TCTGTGAAGC AGTGCTAG

48

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 52 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AATTCTAGCA CTGCTTCACA GAGATGTGGT CTGGTGCTCC CACTGGGTAG TT

52

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 45 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AACTACCCAG TGGGAGCACC AGACCACATC TCTGTGAAGC AGTAG

45

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 49 base pairs
 - (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AATTCTACTG CTTACAGAG ATGTGGTCTG GTGCTCCAC TGGGTAGTT

49

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 52 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTAGACCCGG GATGGCATCC ATGACAGGAG GACAACAAAT GGTAGGCCGT AG

52

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 53 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AATTCTACGG CCTACCCATT TGTTGTCCTC CTGTCATGGA TGCCATCCCG GGT

53

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 13 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CTGCGGCCGC AGT

13

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CTAGACTGCG GCCGCAG

17

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CCAGGTCCAA CTGCAGGTCG ACTCTAGAGG GCACTTCTTC TGGGCCCACG

60

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TATTATCTGT GCGGCCGCCC TCCAGAACCC ATGGCTGCTG GTTGCACTGG

60

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TATTATCTGT GCGGCCGCGC AGCAGGCTCC ACAGTGGTAG

40

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TATTATCTGT GCGGCCGCGG AGGCTCCGTT TCTGGCAG

38

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CGGAGACAGG CCACCGAATT CCTGCCAGAA ACG

33

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CCTCCAGAAA TGCTGAGGCA CAGCACTGAC ACCACTCCTC

40

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GAGCTGGCCA ACATGGGGCA ACTGTCCACG GATTCAGCAG

40

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

AATTCGAGTT CCTAGATTTT G

11

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 11 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AATTCAAAAT CTAGGAATC G

11

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 48 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

AATTCGAGTA CCTAGATTAT GATTTCTGC CAGAACTGA GCCTCCGC

48

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 48 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GGCCGCGGAG GCTCAGTTTC TGGCAGGAAA TCATAATCTA GGTACTCG

48

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 48 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

AATTCGAGTT CCTAGATTAT GATTTCTGTC CAGAACTGA GCCTCCGC

48

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 48 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GGCCGCGGAG GCTCAGTTTC TGGCAGGAAA TCATAATCTA GGAACCTCG

48

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 48 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

AATTCGAGTT CCTAGATTTC GATTTCTGTC CAGAACTGA GCCTCCGC

48

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 48 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GGCCGCGGAG GCTCAGTTTC TGGCAGGAAA TCGAAATCTA GGAACCTCG

48

CLAIMS

1. A composition comprising an isolated DNA selected from the group consisting of:

(a) a DNA encoding a P-selectin ligand protein, said protein comprising an amino acid sequence selected from the group consisting of the amino acid sequence set forth in SEQ ID NO:2 from amino acid 1 to amino acid 402, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 1 to amino acid 310, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 402, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 310, and the amino acid sequence set forth in SEQ ID NO:4; and

(b) a DNA capable of hybridizing under stringent conditions to a DNA specified in (a).

2. A composition of claim 1 wherein said DNA is operably linked to an expression control sequence.

3. A host cell transformed with a composition of claim 2.

4. The host cell of claim 3, comprising a mammalian cell.

5. A process for producing a P-selectin ligand protein, which comprises:

(a) culturing the host cell of claim 3 or claim 4 in a suitable culture medium; and

(b) purifying the P-selectin ligand protein from the culture medium.

6. The process of claim 5 wherein said host cell is co-transformed with a DNA encoding a fucosyltransferase operably linked to an expression control sequence.

7. A composition comprising a P-selectin ligand protein comprising an amino acid sequence selected from the group consisting of the amino acid sequence set forth in SEQ ID

NO:2 from amino acid 1 to amino acid 402, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 1 to amino acid 310, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 402, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 310, and the amino acid sequence set forth in SEQ ID NO:4, said protein being substantially free from other mammalian proteins.

8. The composition of claim 7, further comprising a pharmaceutically acceptable carrier.

9. A composition comprising an antibody which specifically reacts with the P-selectin ligand protein of claim 7.

10. A method of identifying an inhibitor of selectin-mediated intercellular adhesion which comprises

(a) combining a selectin protein with a P-selectin ligand protein comprising an amino acid sequence selected from the group consisting of the amino acid sequence set forth in SEQ ID NO:2 from amino acid 1 to amino acid 402, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 1 to amino acid 310, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 402, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 310, and the amino acid sequence set forth in SEQ ID NO:4, said combination forming a first binding mixture;

(b) measuring the amount of binding between the selectin protein and the P-selectin ligand protein in the first binding mixture;

(c) combining a compound with the selectin protein and the P-selectin ligand protein to form a second binding mixture;

(d) measuring the amount of binding in the second binding mixture; and

(e) comparing the amount of binding in the first binding mixture with the amount of binding in the second binding mixture;

wherein the compound is capable of inhibiting selectin-mediated intercellular adhesion when a decrease in the amount of binding of the second binding mixture occurs.

11. The method of claim 10 wherein said selectin protein is selected from the group consisting of P-selectin protein and E-selectin protein.

12. A method of treating an inflammatory disease which comprises administering a therapeutically effective amount of a composition of claim 8 to a mammalian subject.

13. The process of claim 6, wherein said host cell is also cotransformed with a DNA encoding a GlcNAc transferase.

14. The process of claim 13, wherein said transferase is core2 transferase.

15. The process of claim 6, wherein said fucosyl transferase is an (α 1,3/ α 1,4) fucosyltransferase.

16. P-selectin ligand protein produced according to the method of claim 6.

17. The process of claim 6, wherein said host cell is cotransformed with a DNA encoding a paired basic amino acid converting enzyme.

18. A host cell of claim 4 cotransformed with at least one DNA selected from the group consisting of a DNA encoding GlcNAc transferase, a DNA encoding a fucosyltransferase, and a DNA encoding a paired basic amino acid cleavage enzyme.

19. The host cell of claim 18 wherein said GlcNAc transferase is core2 transferase.

20. The host cell of claim 18 wherein the fucosyltransferase is an (α 1,3/ α 1,4) fucosyltransferase.

21. The host cell of claim 18 cotransformed with a DNA encoding a GlcNAc transferase, a DNA encoding a fucosyltransferase and a DNA encoding a paired basic amino acid cleavage enzyme.

22. The host cell of claim 21 wherein said GlcNAc transferase is core2 transferase and said fucosyltransferase is an (α 1,3/ α 1,4) fucosyltransferase.

23. A method of inhibiting selectin-mediated binding comprising administering a therapeutically effective amount of a composition of claim 8 to a mammalian subject.

24. The method of claim 23 wherein said binding is selected from the group consisting of P-selectin mediated binding and E-selectin mediated binding.

25. A P-selectin ligand protein having P-selectin ligand protein activity.

26. The protein of claim 25 comprising the sequence from amino acid 42 to amino acid 60 of SEQ ID NO: 2.

27. The protein of claim 25 consisting essentially of the sequence from amino acid 42 to amino acid 60 of SEQ ID NO: 2.

28. The protein of claim 25 comprising the sequence from amino acid 42 to amino acid 88 of SEQ ID NO: 2.

29. The protein of claim 25 consisting essentially of the sequence from amino acid 42 to amino acid 88 of SEQ ID NO: 2.
30. The protein of claim 25 consisting essentially of the sequence from amino acid 42 to amino acid 118 of SEQ ID NO: 2.
31. The protein of claim 25 consisting essentially of the sequence from amino acid 42 to amino acid 189 of SEQ ID NO: 2.
32. The protein of claim 25 wherein at least one of the asparagine residues at positions 65, 111 and 292 of SEQ ID NO: 2 have been deleted or replaced.
33. The protein of claim 25 comprising at least one of the tyrosine residues at positions 46, 48 and 51 of SEQ ID NO: 2.
34. The protein of claim 25 which does not bind to E-selectin.
35. An isolated DNA comprising a nucleotide sequence encoding a protein of claim 25.
36. The DNA of claim 35 which further comprises an expression control sequence operably linked to said nucleotide sequence.
37. A host cell transformed with the DNA of claim 36.
38. A process for producing a P-selectin ligand protein, which comprises:
- (a) culturing the host cell of claim 37 in a suitable culture medium; and

(b) purifying the P-selectin ligand protein from the culture medium.

39. A pharmaceutical composition comprising the P-selectin ligand protein of claim 25 and a pharmaceutically acceptable carrier.

40. A composition comprising an antibody which specifically reacts with the P-selectin ligand protein of claim 25.

41. A method of identifying an inhibitor of selectin-mediated intercellular adhesion which comprises:

(a) combining a selectin protein with a P-selectin ligand protein of claim 25, said combination forming a first binding mixture;

(b) measuring the amount of binding between the selectin protein and the P-selectin ligand protein in the first binding mixture;

(c) combining a compound with the selectin protein and the P-selectin ligand protein to form a second binding mixture;

(d) measuring the amount of binding in the second binding mixture; and

(e) comparing the amount of binding in the first binding mixture with the amount of binding in the second binding mixture;

wherein the compound is capable of inhibiting selectin-mediated intercellular adhesion when a decrease in the amount of binding of the second binding mixture occurs.

42. The method of claim 41 wherein said inhibitor inhibits P-selectin mediated binding, but does not inhibit E-selectin mediated binding.

43. The method of claim 42 wherein said P-selectin ligand protein comprises the sequence from amino acid 42 to amino acid 60 of SEQ ID NO: 2.

44. The method of claim 43 wherein said protein consists essentially of the sequence from amino acid 42 to amino acid 60 of SEQ ID NO: 2.

45. A method of inhibiting selectin-mediated binding comprising administering a therapeutically effective amount of a composition of claim 39 to a mammalian subject.

46. The method of claim 45 wherein administration of said composition selectively inhibits P-selectin mediated binding.

47. The protein of claim 25 comprising the sequence from amino acid 48 to amino acid 51 of SEQ ID NO:2.

48. The protein of claim 47 wherein at least one of the tyrosine residues of said protein is phosphorylated.

49. The protein of claim 25 comprising the sequence from amino acid 42 to amino acid 56 of SEQ ID NO:2.

50. The protein of claim 49 wherein at least one of the tyrosine residues of said protein is phosphorylated.

51. The process of claim 15 wherein said fucosyl transferase is selected from the group consisting of Fuc-TIII and Fuc-TVII.

52. The host cell of claim 20 said fucosyl transferase is selected from the group consisting of Fuc-TIII and Fuc-TVII.

53. The composition of claim 1 where said DNA encodes a protein comprising the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 402.

54. The composition of claim 1 where said DNA encodes a protein comprising the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 310.

55. The DNA of claim 35 wherein said protein comprises an amino acid sequence selected from the group consisting of the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 402, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 310, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 60, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 88, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 118, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 189, the amino acid sequence set forth in SEQ ID NO:2 from amino acid 42 to amino acid 56, and the amino acid sequence set forth in SEQ ID NO:2 from amino acid 48 to amino acid 51.

Fig. 1

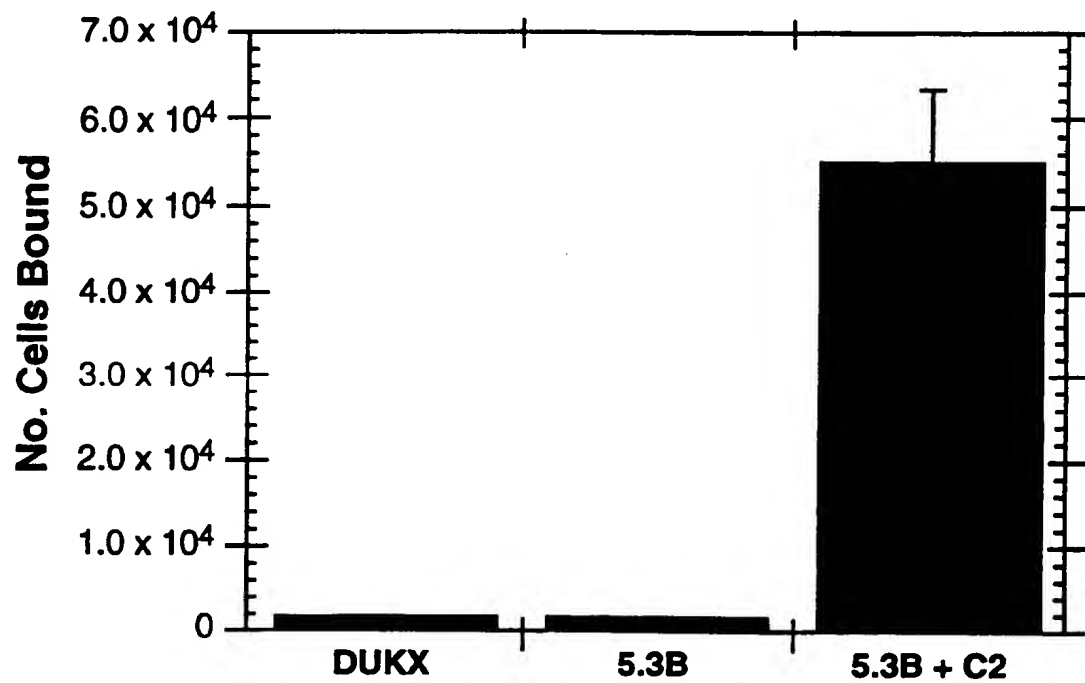


Fig. 2

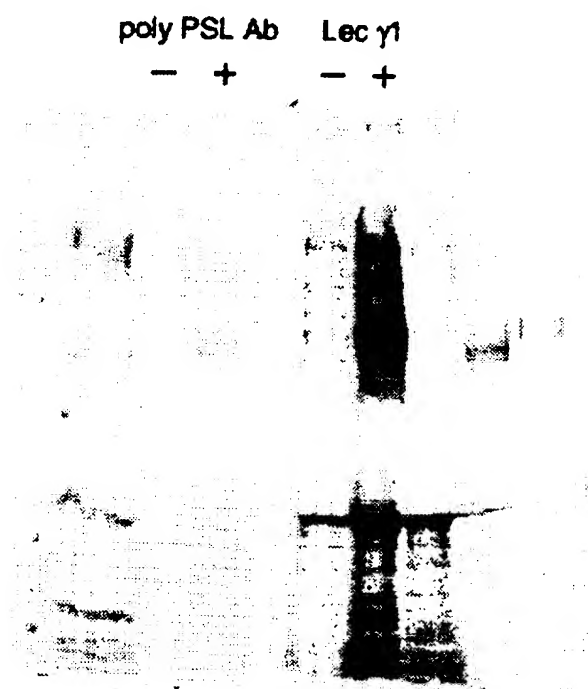
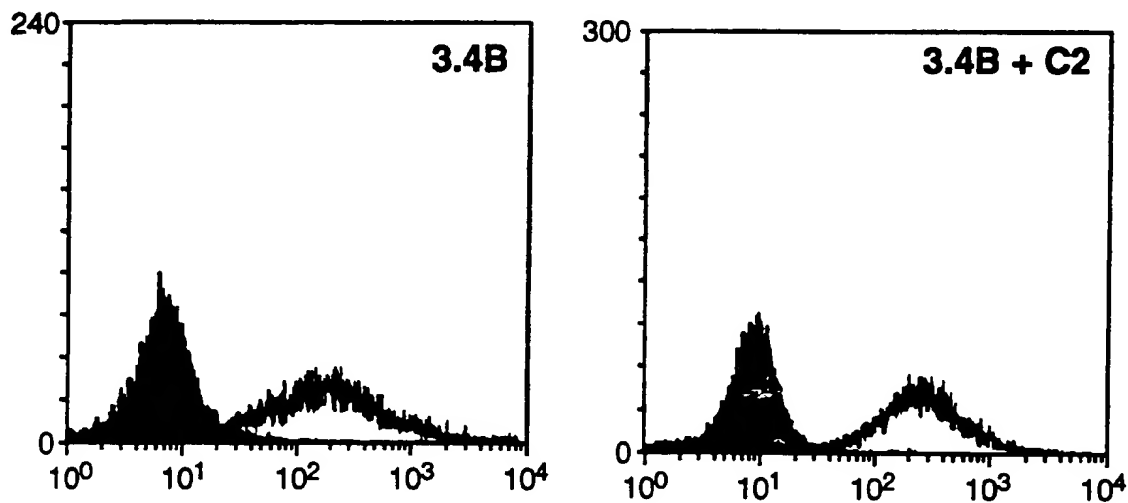


Fig. 3

mAb 275



Lecy1

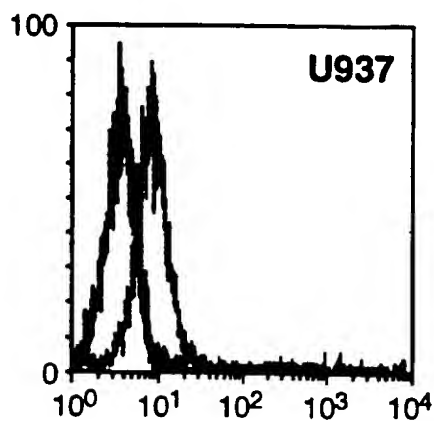
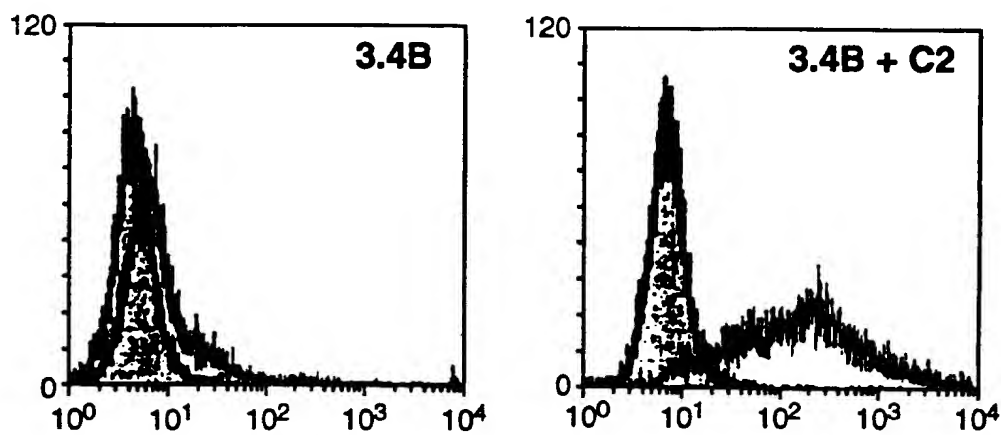


Fig. 4

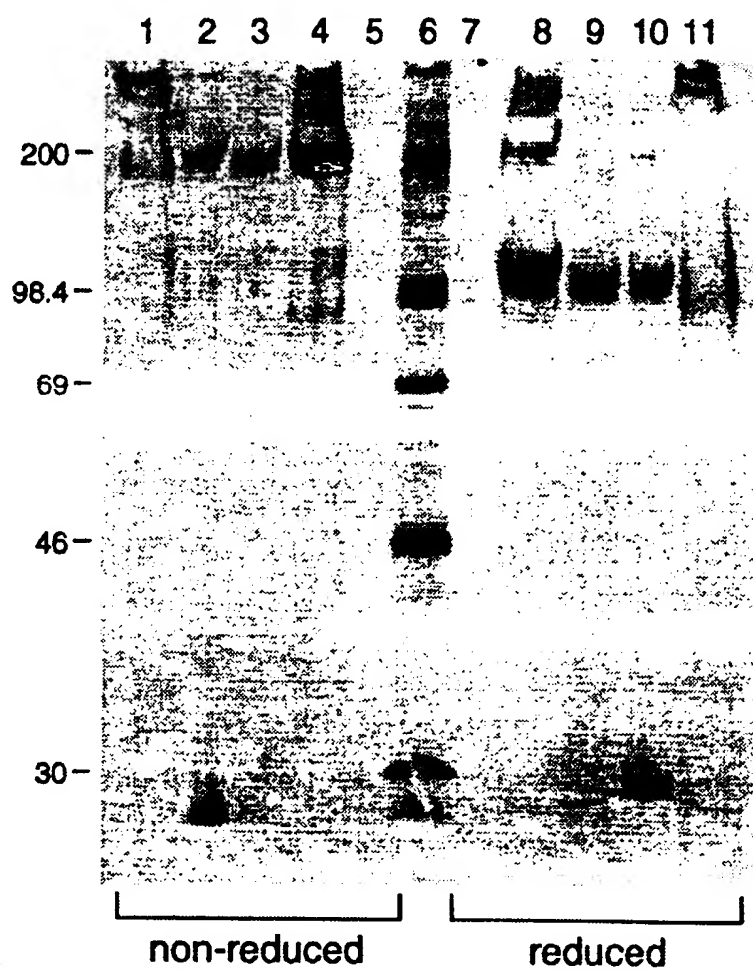


Fig. 5

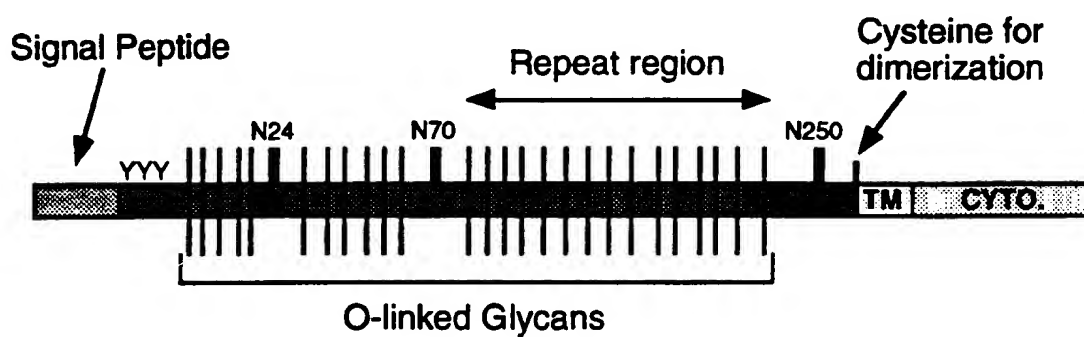
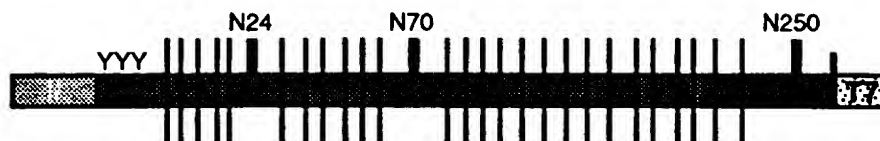
**Soluble "T7" Truncated Form:**

Fig. 6

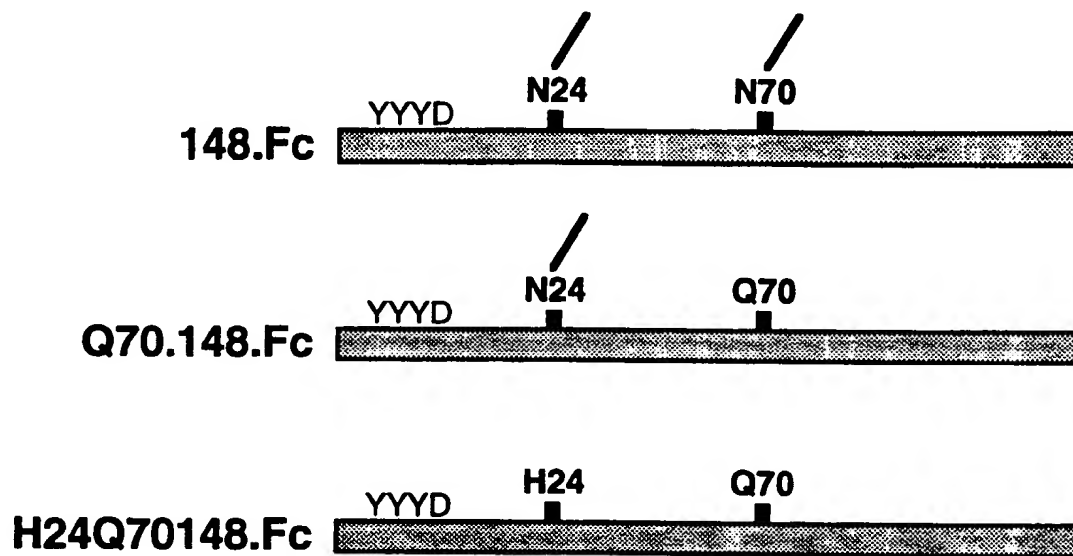
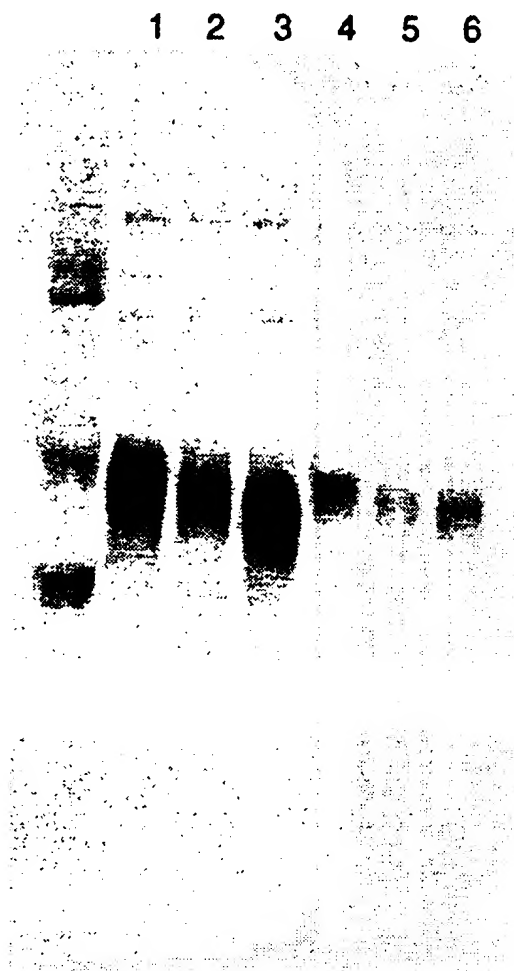


Fig. 7

**³⁵S-Met Labeled***Captured with Protein A*

1. 148.Fc
2. 148.Q70.Fc
3. 148.H24.Q70.Fc

Captured with P-Sel.Fc

4. 148.Fc
5. 18.Q70.Fc
6. 148.H24.Q70.Fc

Fig. 8

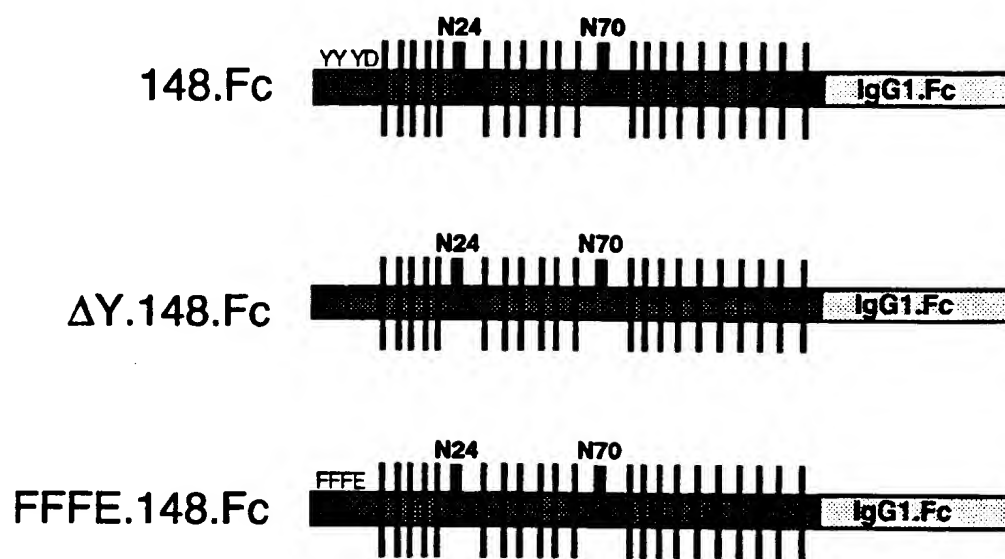
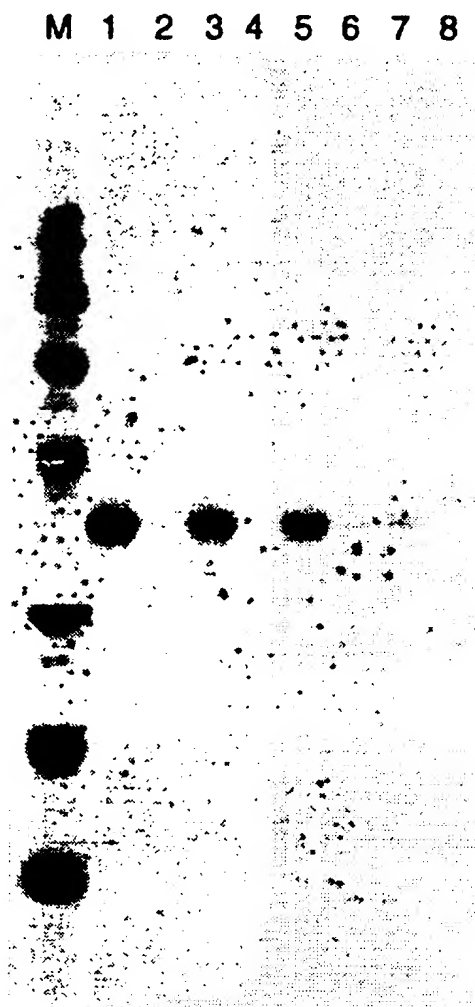
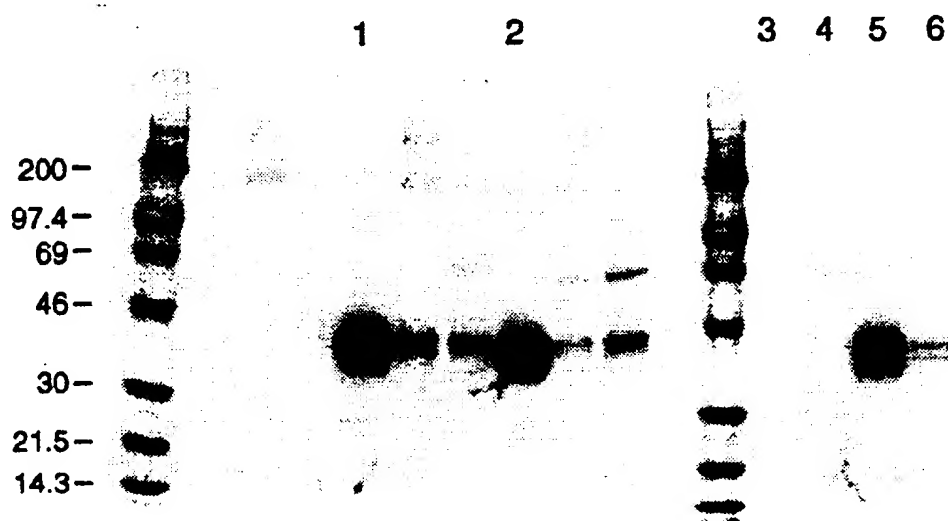


Fig. 9



<u>Sample</u>	<u>Chlorate</u>
M. mw marker	
1. YYY.19Fc	-
2. YYY.19Fc	+
3. FYY.19Fc	-
4. FYY.19Fc	+
5. FFY.19Fc	-
6. FFY.19Fc	+
7. FFF.19Fc	-
8. FFF.19Fc	+

Fig. 10

**³⁵S-Met-Labeled**

1. FYY.19.Fc
2. FFF.19.Fc

³⁵SO₄ Labeled

3. Mock
4. P-Sel.LE.Fc
5. FYY.19.Fc
6. FFF.19.Fc

Fig. 11

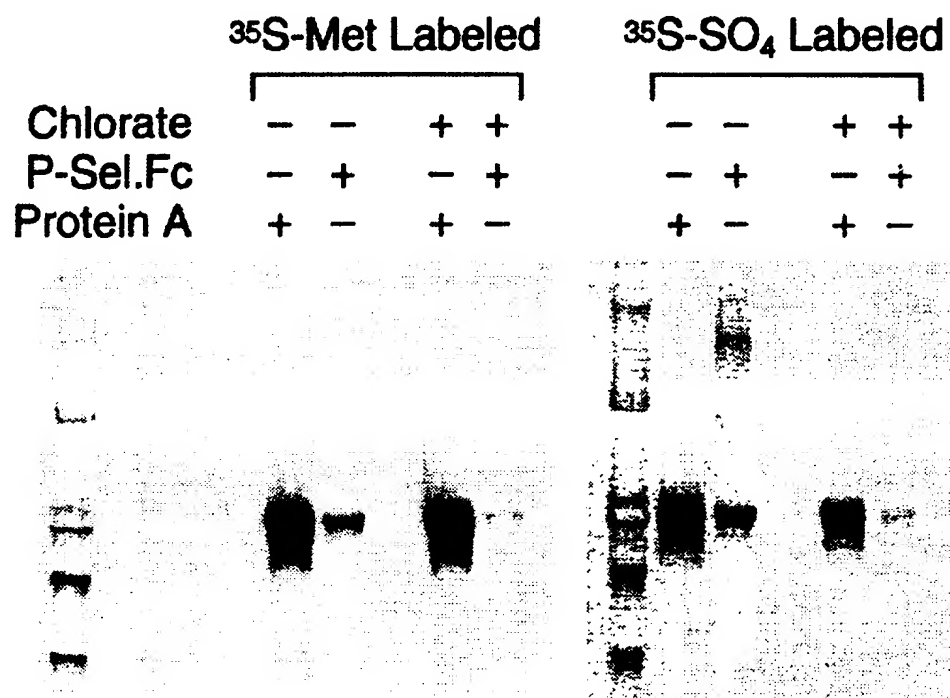


Fig. 12

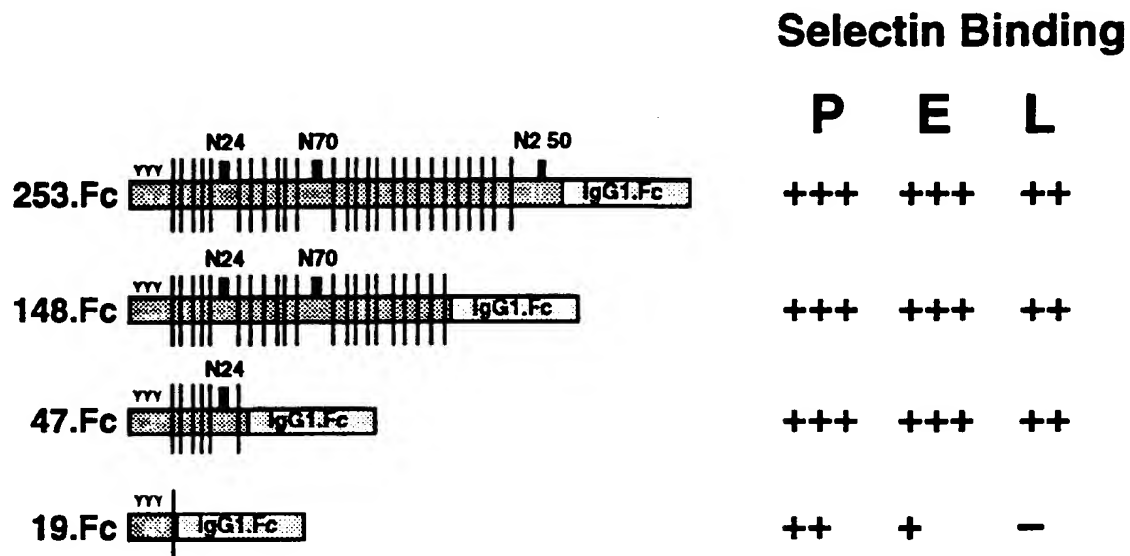


Fig. 13

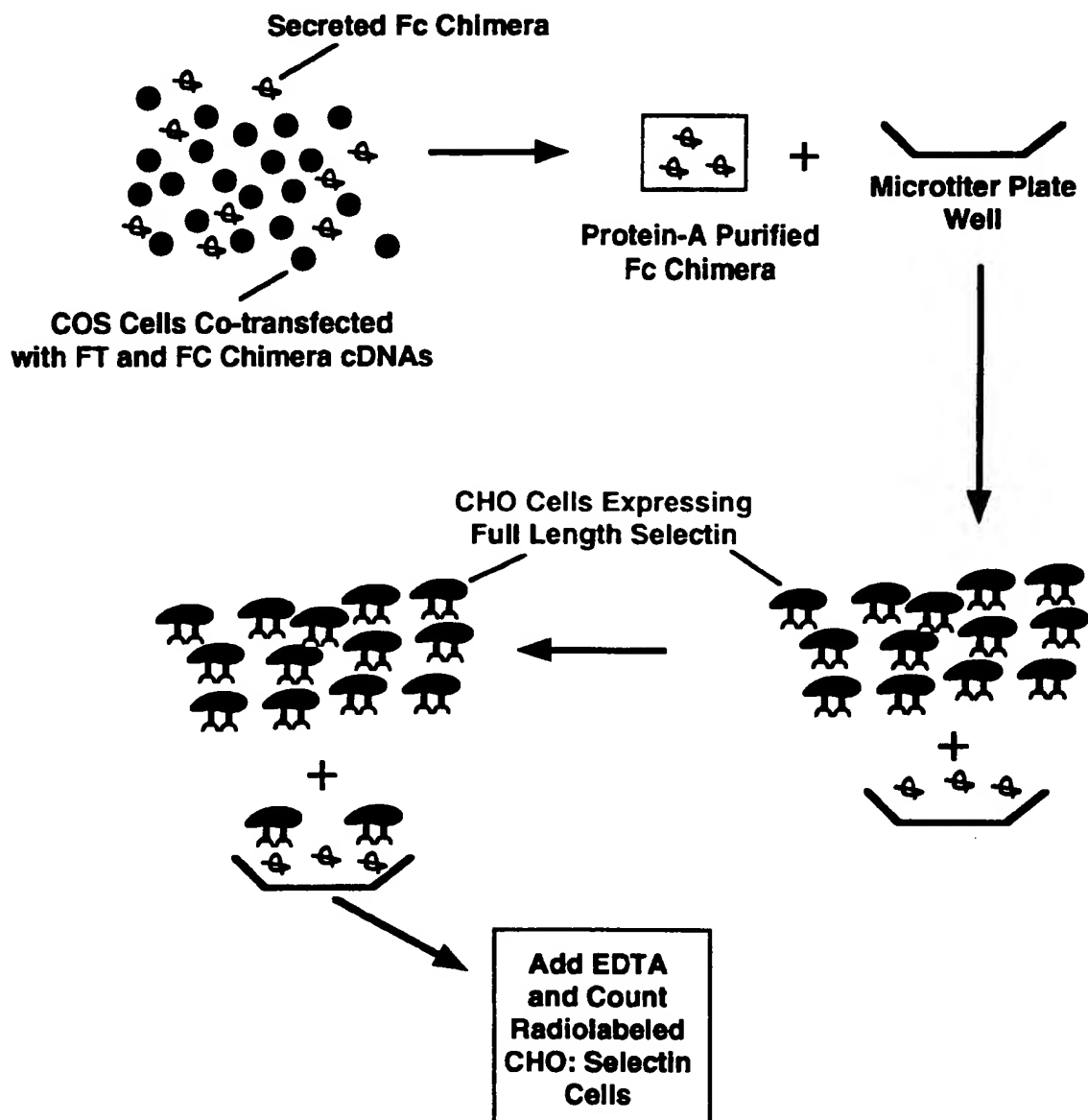


Fig. 14

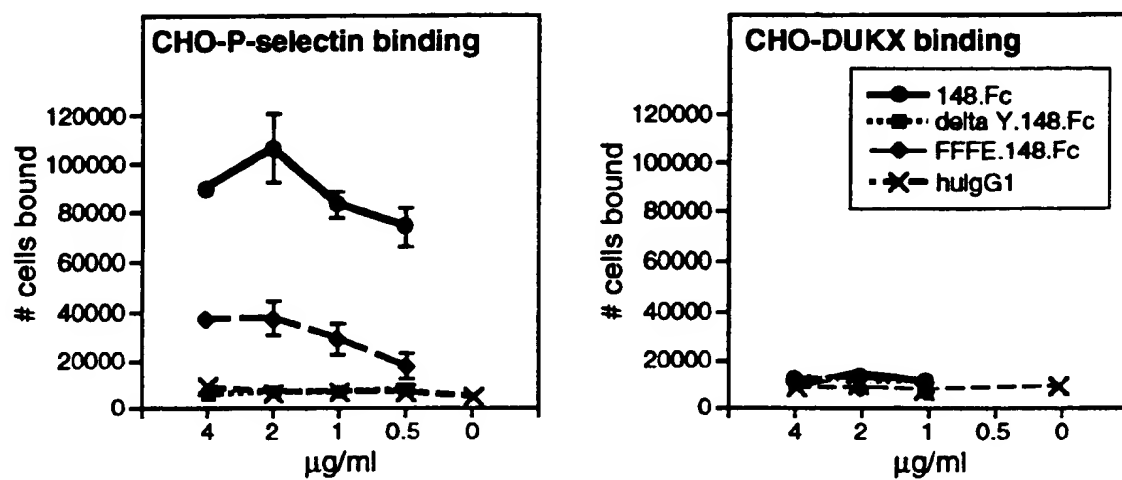


Fig. 15

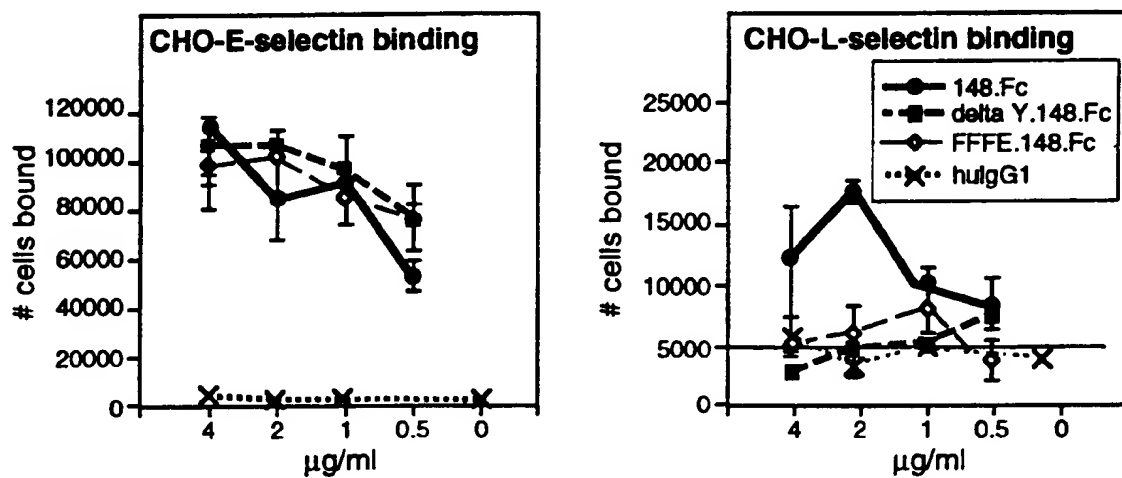


Fig. 16

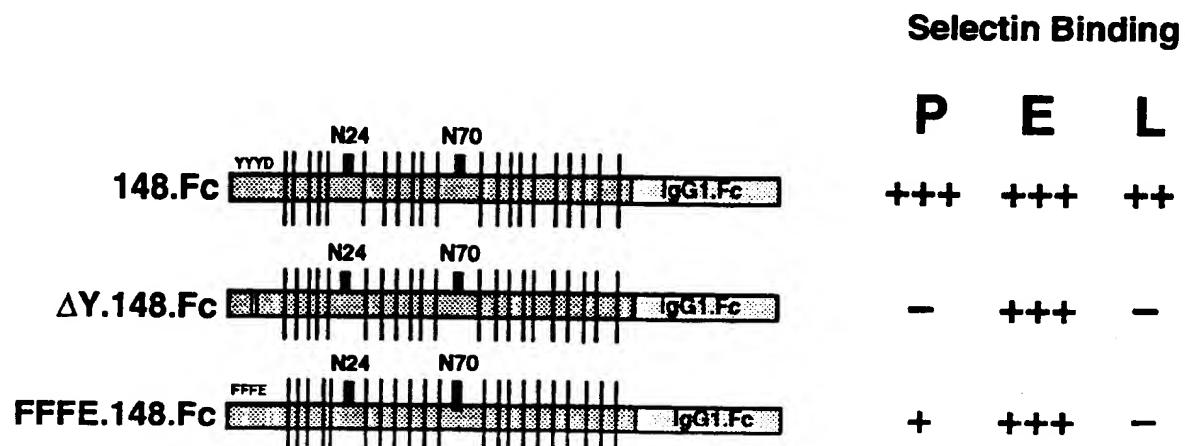


Fig. 17

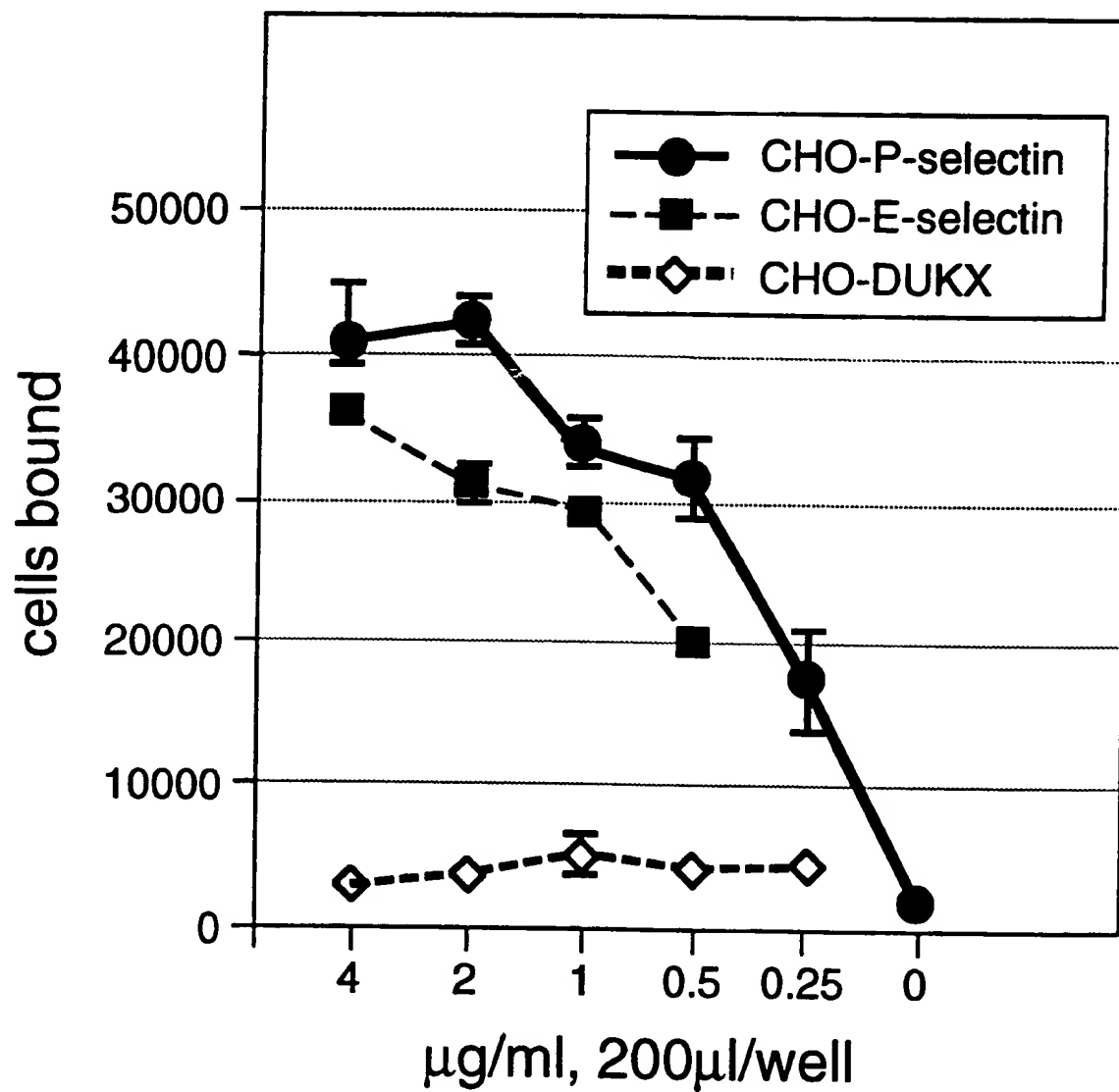


Fig. 18

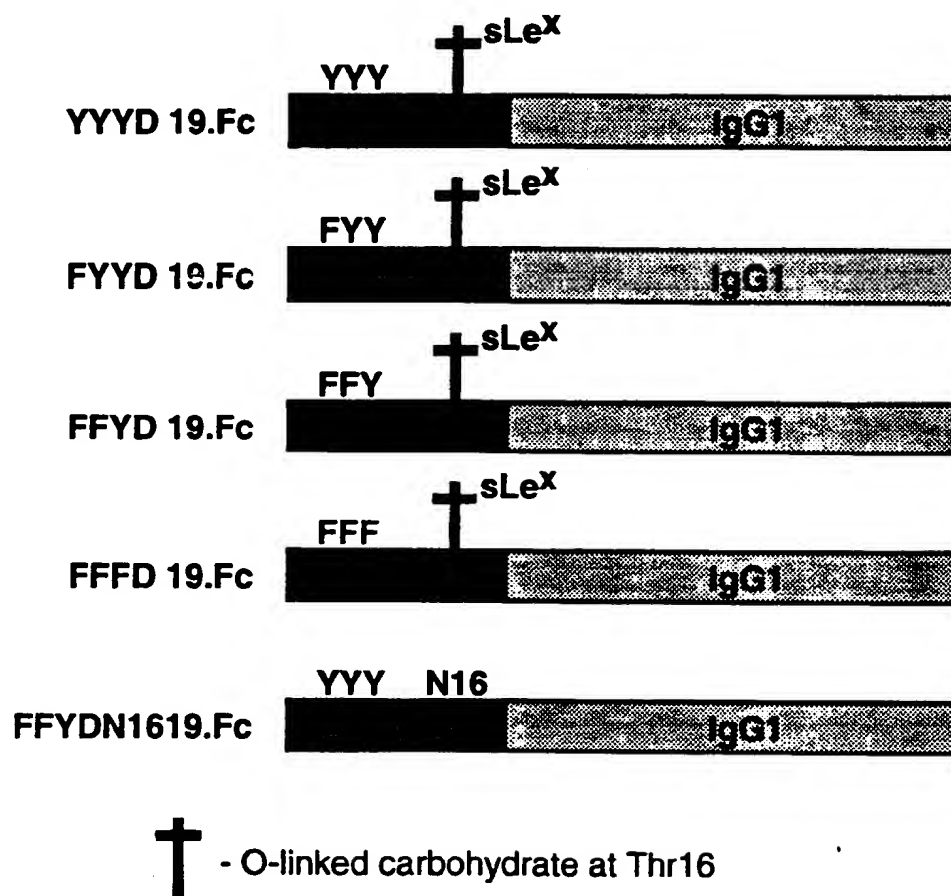


Fig. 19

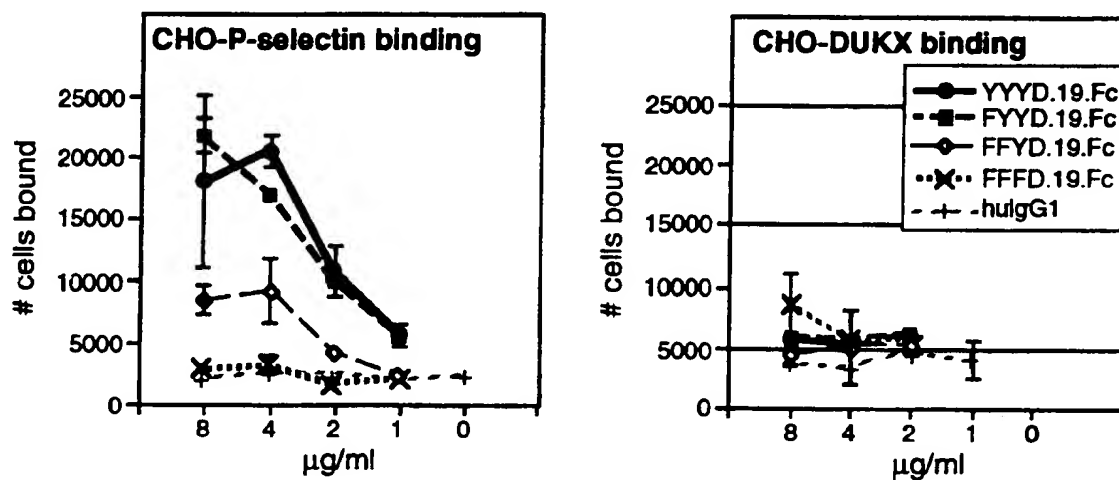


Fig. 20

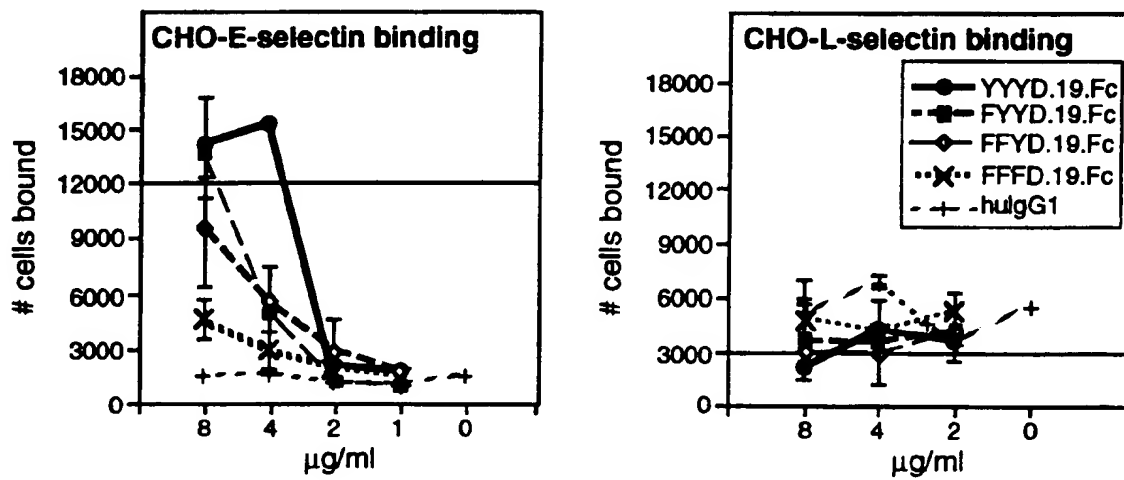


Fig. 21


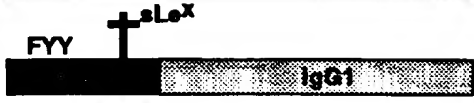



		Selectin Binding		
		P	E	L
YYYD 19.Fc		+++	+	-
FYYD 19.Fc		++	+	-
FFYD 19.Fc		+	+	-
FFFD 19.Fc		+	+	-
FFYDN1619.Fc		-	-	-

Fig. 22

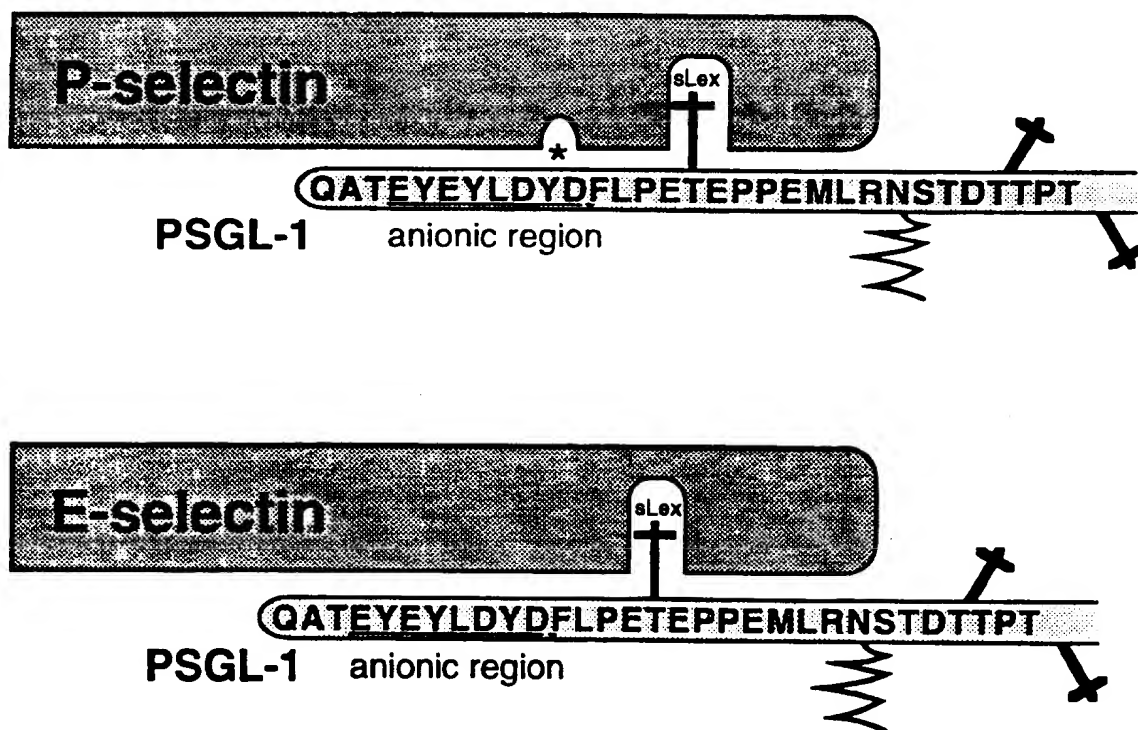


Fig. 23

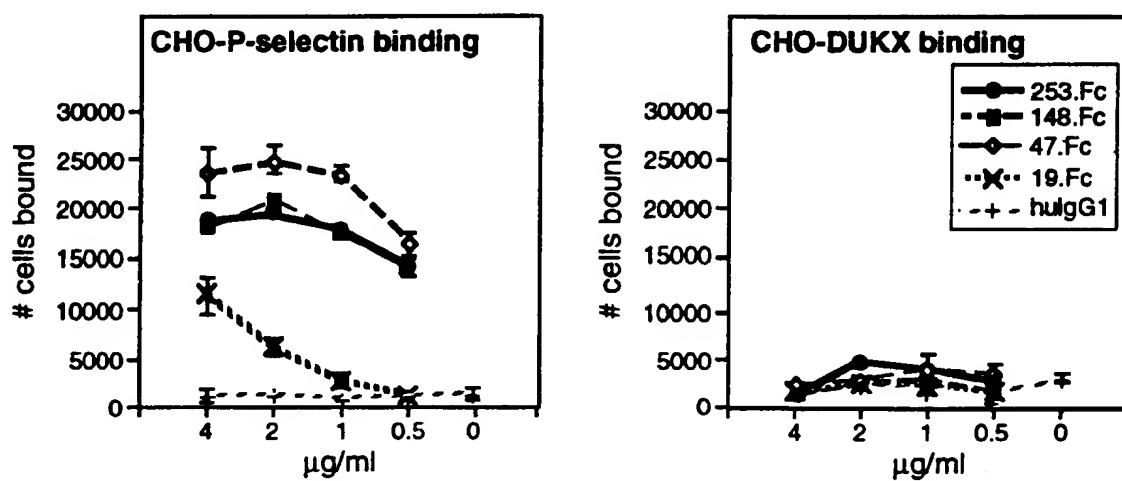


Fig. 24

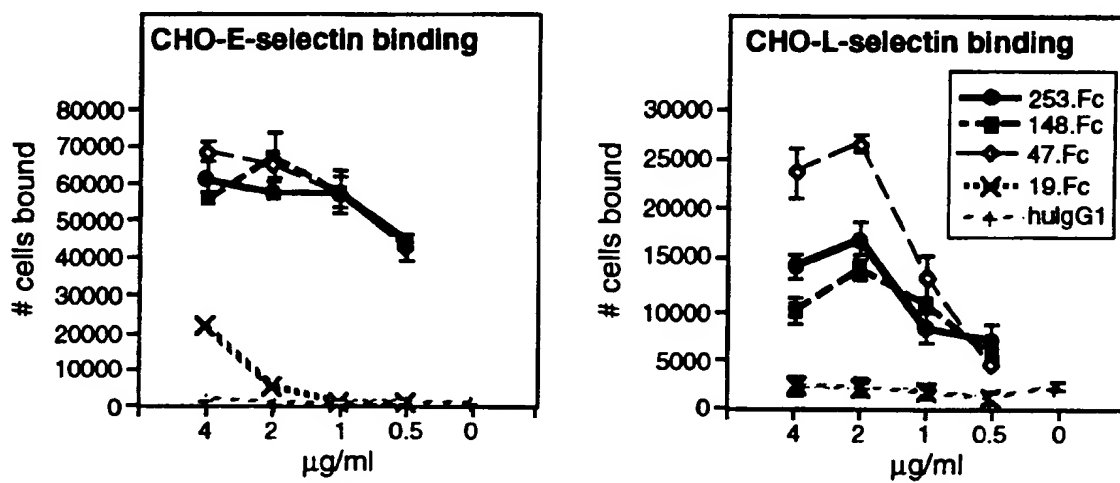
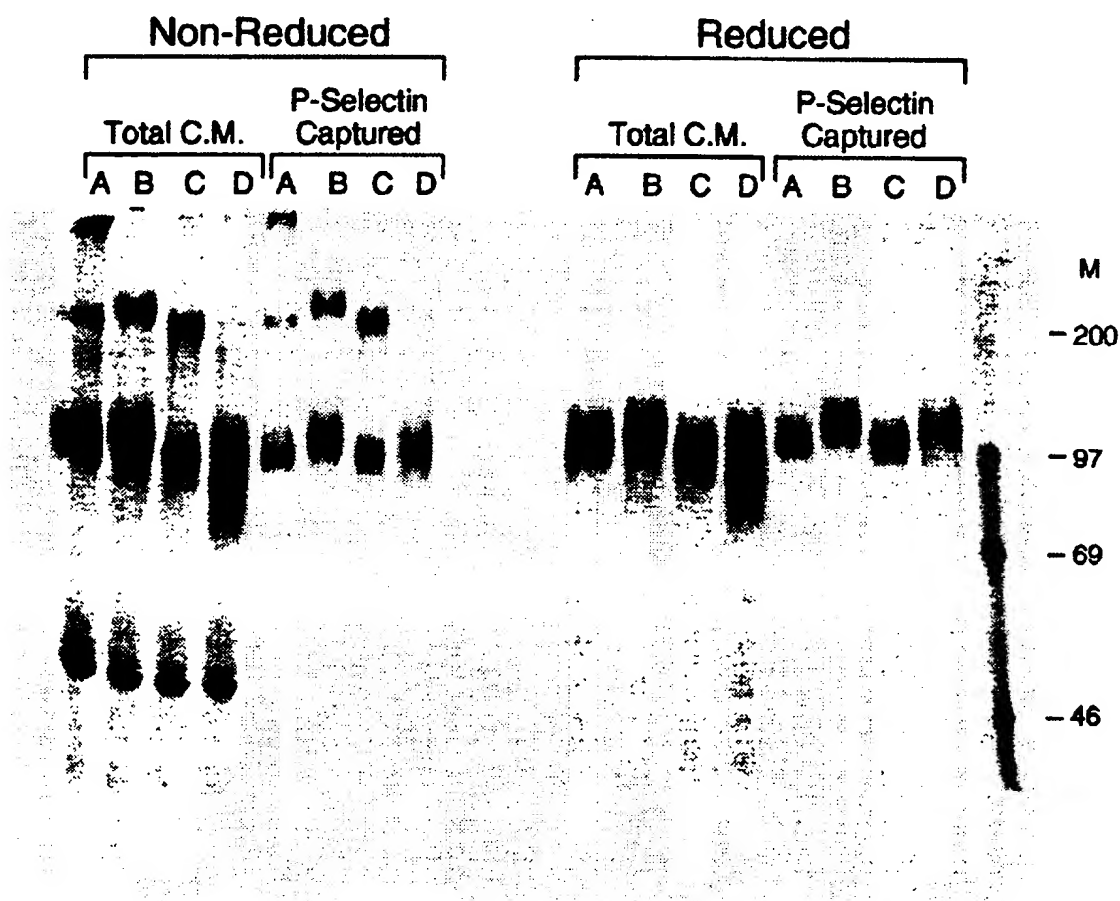


Fig. 25

**Key:**

A - "T7" sPSGL-1

B - " Δ TM" sPSGL-1

C - "I316" sPSGL-1

D - "Qc" sPSGL-1

Fig. 26

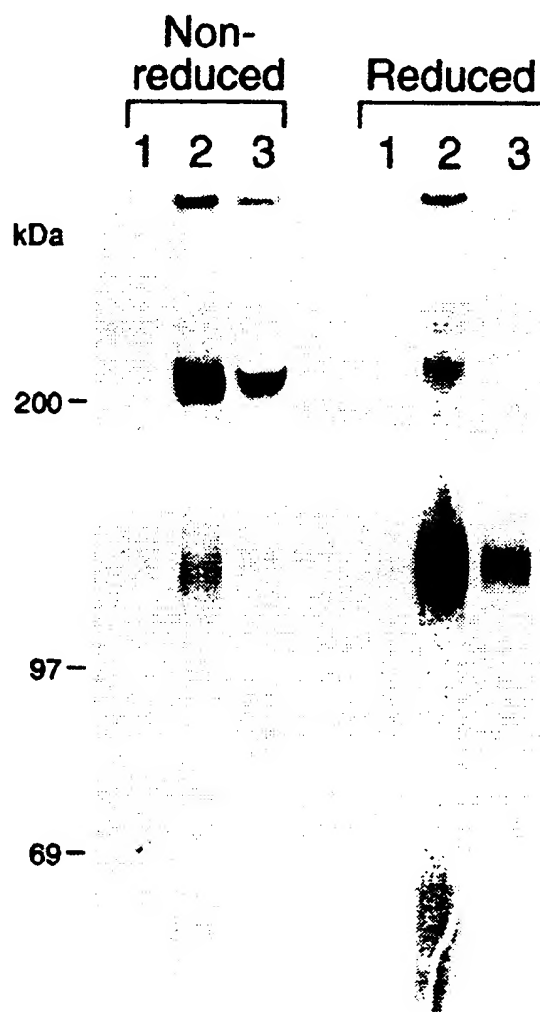


Fig. 27



Fig. 28

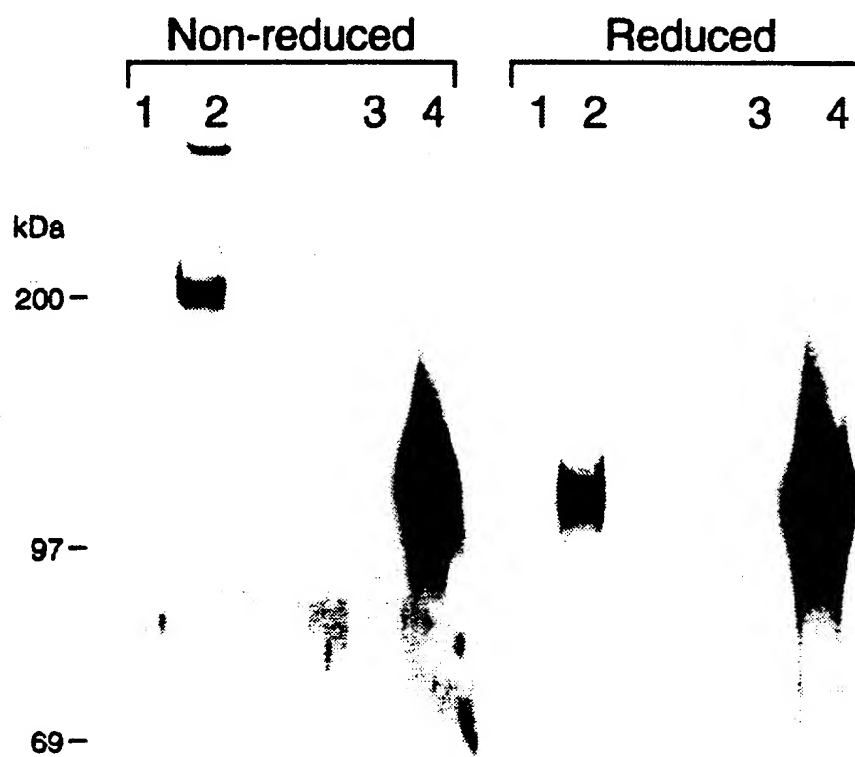


Fig. 29

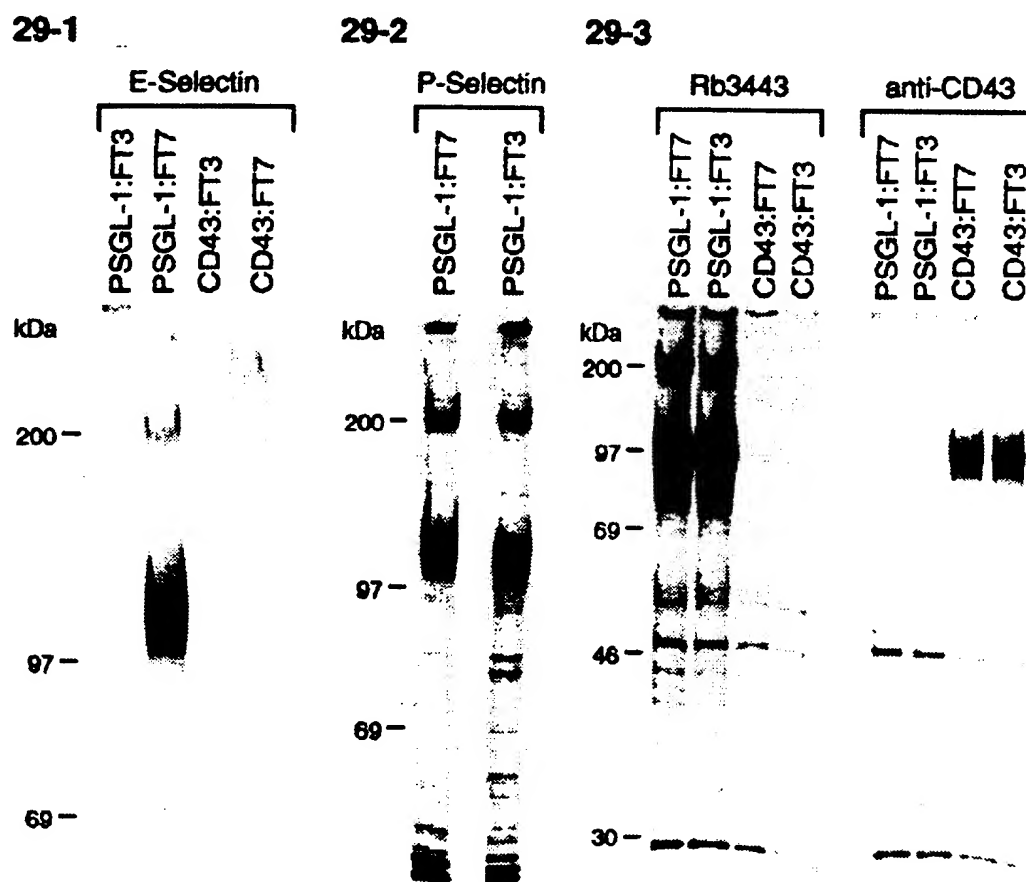


Fig. 30

	<u>E-selectin Inhibition</u>	<u>P-selectin Inhibition</u>
QATEY EYLDYDFLPEC	—	++ (~60 μ M)
TEY EYLDYDF		++
$\begin{array}{c} \text{PO}_3 \quad \text{PO}_3 \\ \quad \\ \text{SYLDYS} \end{array}$	+	+++ (~10 μ M)
$\begin{array}{c} \text{PO}_3 \\ \\ \text{SYLDYS} \end{array}$		+
SYLDYS		—
$\begin{array}{c} \text{PO}_3 \\ \\ \text{SFLDYS} \end{array}$		—
$\begin{array}{c} \text{PO}_3 \quad \text{PO}_3 \\ \quad \\ \text{AcYLDYNH}_2 \end{array}$		+
$\begin{array}{c} \text{PO}_3 \\ \\ \text{AcLDYNH}_2 \end{array}$		—
$\begin{array}{c} \text{SO}_3 \\ \\ \text{SYLDYS} \end{array}$		—



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁶ : C12N 15/12, 9/64, 15/62, C07K 14/705, C12N 15/54, C07K 16/28, C12N 15/57, A61K 38/17, C12N 9/10</p>	<p>A3</p>	<p>(11) International Publication Number: WO 95/30001 (43) International Publication Date: 9 November 1995 (09.11.95)</p>
<p>(21) International Application Number: PCT/US95/04968 (22) International Filing Date: 24 April 1995 (24.04.95) (30) Priority Data: 08/235,398 28 April 1994 (28.04.94) US 08/316,305 30 September 1994 (30.09.94) US (71) Applicant: GENETICS INSTITUTE, INC. [US/US]; 87 CambridgePark Drive, Cambridge, MA 02140 (US). (72) Inventors: LARSEN, Glenn, R.; 112 Maynard Farm Road, Sudbury, MA 01776 (US). SAKO, Dianne, S.; 50 Jamaica Way #6, Boston, MA 02130 (US). CHANG, Xiao-Jia; 59 Commonwealth Park West, Newton Centre, MA 02159 (US). VELDMAN, Geertruida, M.; 60 Woodmere Drive, Sudbury, MA 01776 (US). CUMMING, Dale; 57 Hammond Street, Acton, MA 01720 (US). KUMAR, Ravindra; 533 School Street, Belmont, MA 02178 (US). SHAW, Gray, D.; 551 Barretts Mill Road, Concord, MA 01742 (US). (74) Agent: BROWN, Scott, A.; Genetics Institute, Inc., 87 CambridgePark Drive, Cambridge, MA 02140 (US).</p>	<p>(81) Designated States: AU, CA, JP, MX, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 11 January 1996 (11.01.96)</p>	
<p>(54) Title: NOVEL P-SELECTIN LIGAND PROTEIN (57) Abstract A novel P-selectin ligand glycoprotein is disclosed, comprising the amino acid sequence set forth in SEQ ID NO:2 or by the amino acid sequence set forth in SEQ ID NO:4. DNA sequences encoding the P-selectin ligand protein are also disclosed, along with vectors, host cells, and methods of making the P-selectin ligand protein. Pharmaceutical compositions containing the P-selectin ligand protein and methods of treating inflammatory disease states characterized by P-selectin- and E-selectin-mediated intercellular adhesion are also disclosed.</p>		

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INTERNATIONAL SEARCH REPORT

Internat Application No

PCT/US 95/04968

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N15/62 C12N15/54 C12N15/57 C12N9/10
 C12N9/64 C07K14/705 C07K16/28 A61K38/17

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CELL, vol. 75, no. 6, 17 December 1993 CELL PRESS, CAMBRIDGE, MA, US;; pages 1179-1186, D. SAKO ET AL. 'Expression cloning of a functional glycoprotein ligand for p-selectin' cited in the application	1-7,9, 10,15, 18,20, 25,26, 28,35, 38,40, 41,47, 49,53-55
Y	the whole document --- -/--	13,14, 21,22

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

26 September 1995

Date of mailing of the international search report

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	WO,A,92 19735 (GENENTECH, INC.) 12 November 1992 see page 17, line 10 - line 37; claims 1-38 ---	1-6,8-10

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

1. national application No.

PCT/US 95/ 04968

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 12,23,24,45,46 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat'l Application No

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